

Prevention of spina bifida—the parents' choice

THE problem of prevention and treatment of the congenital malformation spina bifida is in the news again. An all-party motion in the British House of Commons to make prenatal screening for the condition available to all expectant mothers as soon as possible has been signed by over 200 MPs. The screening would involve a blood test, followed, if positive, by amniocentesis (drawing off amniotic fluid) to provide a more sensitive test, followed by offer of abortion if the foetus were found to have spina bifida. Amniocentesis is also of value in the detection of chromosome anomalies such as Down's syndrome and a number of other disorders. Some strong views and some incorrect statistics have been flying around this past month; maybe it will be of value first simply to record some of the basic facts.

The incidence of the neural tube defects, anencephaly and spina bifida varies with race and place. In south-east England the incidences are one in 800 births for anencephaly and one in 600 for spina bifida. But in Wales, Scotland and Ireland the incidence is roughly double this, whereas in African and Mongoloid races the incidence can be less than half that in south-east England. The reasons for this variation are unknown—a different gene pool, soft water or the consumption of blighted potatoes have all been suggested as causes, but so far without confirmation.

If parents have had an affected child the incidence in future siblings is around 4%. There is evidence that both genetic and environmental influences are at work; in most series, consanguinity in the parents is found to be commoner than in the average marriage, monozygotic twins show a higher concordance rate than dizygotic twins and incidence is higher in lower socio-economic groups.

The proposed screening test is an assessment of the level of alpha-foetoprotein in maternal serum. This alpha-immunoglobulin, of uncertain function, is synthesised by some foetal cells and the concentration in foetal serum rises from the 6th to the 14th embryonic week, after which it declines to zero at birth. In neural tube defects the amniotic fluid level is considerably greater than normal and this is reflected in a significant but lesser elevation of the level in the maternal serum. Unfortunately a number of other conditions can give unusually high alpha-foetoprotein levels in the amniotic fluid, such as twinning, foetal death, rhesus immunisation and some other congenital deformities, although it has recently been

shown that study of the cells in the amniotic fluid can discriminate between neural tube defects and other causes of raised alpha-foetoprotein. Other tests such as X-rays, ultrasonics and foetoscopy (direct inspection of the foetus through a fibre-optic instrument) can add to the accuracy of diagnosis, so the likelihood of a false positive result in experienced centres is small. The risk to the mother also seems to be very small, apart from the possibility of rhesus sensitisation. There is a risk estimated at 1–2% to the foetus, mainly of induced abortion, though a recent study showed no significantly increased incidence of abortion.

The outlook for a child born with a neural tube defect varies from certain death in anencephaly to mild weakness of the lower limbs when the defect is minimal. A typical case, if left untreated, will die of infection in due course (though there is occasional survival). With surgery, survival is usual, which is why this was undertaken for most cases from the late 1950s. But there is usually considerable handicap (paralysis of legs, incontinence and deformity—sometimes hydrocephalus and mental deterioration). Most parents who have struggled to look after such a child are prepared to have a prophylactic abortion rather than repeat the experience. The often gloomy outlook has led some doctors to advocate non-treatment of all except mild cases, and Robert Zachary, Professor of Paediatric Surgery at Sheffield University, has recently alleged in the *British Medical Journal* that some are going further than this and practising a mild form of euthanasia. He considers, and an unknown proportion of sufferers from the condition and their parents would agree, that even a restricted life is decidedly better than no life at all.

There thus seems every reason for supporting a much wider availability of screening programmes, not just to restrict the potential for suspicions of euthanasia and not just to reduce unnecessary suffering, but also to give parents a chance to make a calmer and more rational decision at an early stage. Screening is expensive and needs staff training; it will therefore need to be provided on a selective basis, and parents will have to realise that a decision to undergo amniocentesis should not be taken unless they are prepared to go through with an induced abortion if the indications are unfavourable. Association by the Roman Catholic Archbishop of Glasgow of screening programmes with Nazi sterilisation programmes is one way of ensuring that decisions are not taken calmly. □



Benefit-cost analysis and the linear hypothesis

Alvin M. Weinberg, Director of the Institute for Energy Analysis at Oak Ridge, Tennessee warns against a common pitfall of administrators when trying to make cost/benefit analyses of new technologies

ECONOMICS is indeed the dismal science. This was brought home to me as I read the recent report (BEIR-II) of the US National Academy of Sciences, 'Considerations of Health Benefit-Cost Analysis for Activities Involving Ionising Radiation Exposure and Alternatives'. This is a sequel to the 1972 report on Biological Effects of Ionising Radiation (BEIR-I), a study that has been as widely misinterpreted and misused as any the National Academy has issued.

Benefit-cost analysis is simple in principle. In judging whether mass x-ray screening or nuclear power or therapeutic x-rays are worthwhile, one tots up the benefits—early detection of cancers, cheaper electricity, extended life span—and the costs: higher incidence of cancer or genetic disease induced by radiation. If the benefits exceed the costs, the technology is acceptable; if not it is unacceptable—QED.

Unfortunately the measures of cost (human health) and benefit (electricity) are not the same; or much worse, where the exposures are very small, the very question of estimating costs becomes essentially trans-scientific. At least at our present stage of scientific knowledge, and possibly forever, we cannot estimate the carcinogenic or genetic effect of 1 mrem of ionising radiation.

But the economists, not to say administrators, demand a neat cost/benefit calculus. This requires that a common measure, dollars per life, be used for costs and benefits; and this further requires that the vast uncertainties, even uncertainties in principle, as to the effects of low levels of ionising radiation, be obliterated by a stroke of administrative science.

That stroke of administrative scientific ingenuity is the assumption that the linear hypothesis holds down to zero dose (in other words that the biological effect is proportional to dose regardless of the size of dose or the rate of exposure), and the inevitable introduction of the unit 'person-rem'—the product of the number of individuals exposed and the dose in rems to each individual. Having brushed aside the profound illogicalities in the use of the person-rem as an estimator of actual biological damage, the economists and administrators have made the problem tractable: assign so many dollars per person-rem as the cost, so many dollars per life saved, and the cost/benefit calculus is saved.

That nonsense, of course, lies in the use of the person-rem as an estimator of damage. Curiously, the body of the BEIR-I report cautions against so using the person-rem; but the summary, possibly written by someone other than the authors of BEIR-I, in effect uses the person-rem to place bounds on the number of cancers induced by very low level exposure of large populations. BEIR-II takes up where BEIR-I left off, and unfortunately does not re-examine the validity of the original assumption concerning the use of the person-rem as an estimator of damage.

Nevertheless, in a perceptive paragraph in what I thought was the report's best chapter 'Legal and institutional aspects of using benefit-cost analysis to control ionising radiation', one reads "Serious question should be given to the adoption of alternatives to traditional economic benefit-cost analysis for such regulatory decision-making . . . One possible alternative is an appropriately comprehensive cost-effectiveness analysis . . . (that) requires the articulation of objectives, the weighing of the alternative means to achieve these objectives, and the

selection of the least costly approach". Thus, instead of performing magical balancing acts with largely indeterminate costs weighed against incommensurable and equally indeterminate benefits, concede the arbitrary, political nature of the objective at the outset—agree on an appropriately small level of radiation insult that is tolerable, and then go about devising a technology that will meet that objective as cheaply as possible.

A hint as to what the "appropriately small level of insult" should be was given in the 1958 report of the Ad Hoc Committee of the National Committee on Radiation Protection (The Friedell Report *Science*, **131**, 482; 1960). Since the biosphere has evolved in the presence of a natural background of radiation, Friedell asked whether man-made radiation ought to be judged tolerable if it were 'small' compared to this background. This very sensible suggestion was never followed up—instead, we introduced the nonsensical person-rem and strict linear hypothesis, and continued to juggle indeterminate costs and benefits.

To be sure, the definition of 'small' compared to natural background is arbitrary, but at least the arbitrariness is explicit. H. I. Adler, the former director of the Biology Division of Oak Ridge National Laboratory, has suggested (*Health Physics*, in press) that 'small' be taken as the standard deviation of the population-weighted natural background. This amounts, in the United States, to about 20 mrad per year of gamma radiation, and somewhat miraculously, is close both to the EPA emission standard for the nuclear fuel cycle and the NRC standard for emissions from nuclear power plants. The suggestion has been taken up by the American Physical Society's Committee on the Nuclear Fuel Cycle; they point out that if Adler's standard were adopted, then the actual exposures will average to something small compared to the variability in background.

Adler's suggestion amounts to cutting the Gordian knot: proving or disproving the linear hypothesis at very low exposure. The observed incidence of leukemia at Nagasaki persuades me that linearity, with slope determined by the incidence of leukemia at *high* dose, is inconsistent with the data. Others have looked at various bits of data and insist they see straight lines that go through the origin. But the bald fact is that linearity, upon which the apparatus of cost/benefit analysis for ionising radiation now largely rests, is, for low doses, simply an unproven, probably unprovable hypothesis. I think something like Adler's idea is a far more logical approach to standard setting.

I wax rather vehement because the use of person-rem as an estimator of damage at low dose has badly warped the nuclear debate, and it may have the same effect on the debate over the use of diagnostic x-rays. Thus most of the estimated casualties from a bad nuclear accident are found in the large dispersed population exposed to small doses of radiation. In the original Rasmussen report on reactor safety, credit was therefore given for low doses and dose rates (the strict linear hypothesis was rejected), but the critics insist on using the strict linear hypothesis. BEIR-II, despite the disclaimer previously quoted, recommends that "national policies involving activities such as nuclear power production and medical uses of radiation should be guided to the extent possible by health benefit-cost analysis"; and throughout the report strict linearity is assumed. Why could not this second BEIR report help undo the vast damage caused by the widespread misunderstanding of the first BEIR report, and state, clearly and explicitly, that the use of the person-rem as an estimator of damage is bad science, and this leads to bad policy? I believe BEIR-II must be judged a failure in not having taken the opportunity to correct this unfortunate and widespread misuse of its predecessor report. □

Vietnam's dioxin problem

Much of Vietnam was sprayed with herbicides during the war. **Alastair Hay** reports on local research to assess the damage caused.

MANY times has the United States been accused of using Vietnam as a testing ground for some of the more sophisticated techniques of modern warfare. Indeed, that accusation featured prominently in much of the Vietnamese propaganda material produced during the last ten years of the war. The Vietnamese, however, were not the only accusers: United States scientists were among some of the more vocal critics of their country's military policies. Many of them made it known that they deplored such practices as the use of chemical defoliants; the implementation of weather-modification techniques; the development of plastic-pellet bombs, the intention of which was to produce shrapnel undetectable by x-rays; and the training of dolphins and porpoises to place limpet mines. All these they considered to be perversions of scientific endeavour.

Their opposition, however, had little effect upon the United States' military

activity. In many instances, information was made public only long after the event and the United States always denied emphatically that Vietnam was used as a test site for the development of military technology. But it was the first country in which defoliants were used on a large scale purely to achieve a military objective.

Originally research into 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) and related herbicides had been carried out during the Second World War. The British later tested their effectiveness in small-scale operations during the Malayan 'emergency' of the 1950s. But it was the success of herbicide evaluation trials in South Vietnam, Thailand, Hawaii and Puerto Rico which led to the chemicals being employed on a vast scale in Vietnam. According to a US National Academy of Sciences report of 1974 on 'Herbicides in South Vietnam', more than 10% of the inland forests, 36% of the

mangrove forests, 3% of cultivated and 5% of 'other' land was sprayed "one or more" times.

NAS estimates of the total volume of herbicide dropped on Vietnam are 17.5 million gallons, of which 11.25 million gallons was in the form of 'Agent Orange' (a 50:50 mixture of the n-butyl esters of 2,4-D and 2,4,5-T). Criticism of the use of these herbicides on ecological grounds had little impact on the actual spraying missions. With powerful advocates in the American Chemical Society defending the use of herbicides as a more 'humane' form of warfare, it required more than a concern for ecology to bring the spraying to an end.

When the US Department of Health, Education and Welfare published a report in 1969 announcing that 2,4,5-T was teratogenic in rats and mice, the herbicide programme diminished. The report was confirmed by a later independent study which established that commercial preparations of 2,4,5-T contained a highly toxic contaminant, 2,3,7,8-tetrachlorodibenzo-p-dioxin (dioxin), a teratogen at the μg per kg level in experimental animals.

Sample stocks of Agent Orange retrieved from Vietnam for analysis by the manufacturer, the Dow Chemical Co., revealed dioxin to be present in concentrations of 0.05–47 ppm. On the basis of these figures, it has been estimated that at least 100 kg of dioxin was deposited on Vietnam.

Not just a matter for science

The damage caused by herbicides is a subject of some concern to the Vietnamese authorities, and is an area of investigation for some of the country's scientists and clinicians. If the dioxin problem in Vietnam was simply one of scientific investigation, more published information would be available by now for scrutiny. But some authorities in Vietnam fear that information on the herbicide damage will be used by the military in the United States to refine its chemical warfare techniques.

Dr Tran Tri, one who voices that anxiety, is in charge of scientific relations with western countries. He says that the dioxin issue is a "complicated" matter and adds: "Of course, there is a scientific aspect to this problem which the Americans have used; our scientists, however, will not use the results in the same way". Tri wants Vietnamese scientists to do much of the general investigation on dioxin. Because of the sensitivity of the subject, he states categorically that only very few of those foreign scientists who have expressed an interest will be able to participate.

Not all Vietnam's scientists agree with Tri's interpretation. Some feel sure that the United States has more

VIETNAM occupies a long narrow strip of Indo-China. The South China Sea is the eastern boundary and a series of mountain ranges in the west act as a natural frontier with Laos and Kampuchia.

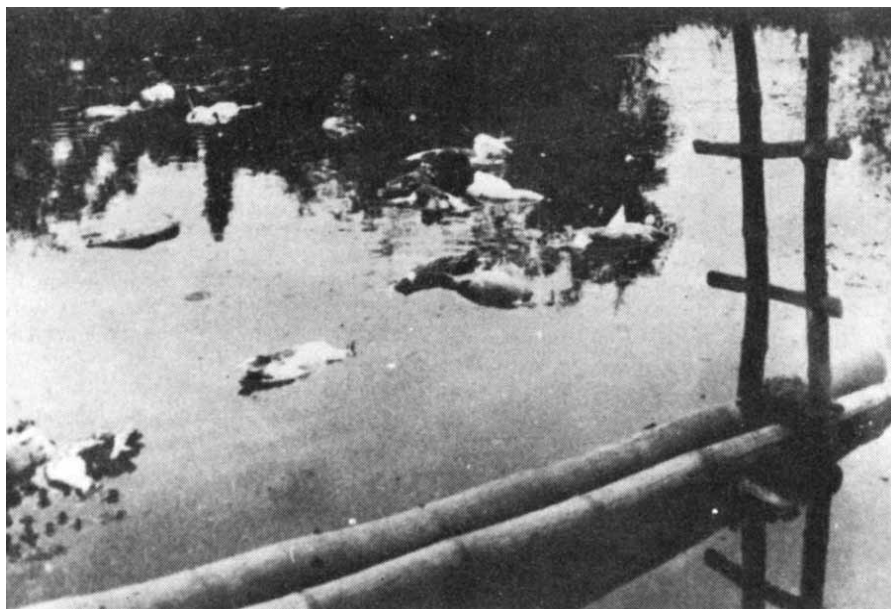
Most of Vietnam's population estimated at 50 million reside in the coastal plain and the delta regions of the Red River and the Mekong. Hanoi, the capital city, houses about 1.3 million people; Ho Chi Minh City has about 3.5 million. Five sixths of the country's population is, ethnically speaking, true Vietnamese; some 39 minority groups, of which the "Montagnards" and Chinese settlers are the largest, account for the other sixth.

Vietnam has had various occupation forces in the past two millennia. China kept her as a province from 111 BC until ousted by a national Vietnamese army in 905 AD. Subsequent attempts by successive Chinese emperors to recolonise Vietnam met with no success and the country enjoyed independence until occupied by French troops in 1858. French suzerainty in Vietnam ended in 1954, only to be partially replaced by an American presence in the south of the country. The United States, however, had a much shorter stay than France. American involvement in Vietnam finally ended with the fall of Saigon (now Ho Chi Minh City) to Communist troops in April 1975.

The war in Vietnam has exacted a heavy toll on the country with most of her resources being devoted to the war effort. The result of this is that the per capita income is exceedingly low and United Nations sources confirm that Vietnam



qualifies for urgent development aid. The distribution of aid provided by foreign donors is therefore the subject of some scrutiny. Vietnam does, however, ration food to ensure fair distribution. She has a free education programme and places great emphasis on preventive medicine. These are strategies which are generally welcomed by the international development agencies.



Medical and Scientific Aid to Vietnam

Vietnamese poultry killed by toxic chemicals

than enough information on herbicides. Scientists in the West working on 2,4,5-T and dioxin are of the same opinion. They point to the herbicide trials undertaken in South-East Asia in the 1960s, and to those run by the US Air Force in Florida from 1962 to 1974. Reports of the Florida experiments—which also contained information on the toxicity and biological degradation rates of dioxin—were declassified in October, 1976, to enable the Italian authorities to make use of them in dioxin decontamination work following the accident at Séveso.

Two of those most involved with the herbicide issue in Vietnam are a cytologist Dr Bach Quốc Tuyen; and Professor Ton That Tung, a surgeon and director of the Viet Duc hospital. Tuyen is in charge of the cytogenetics laboratory at the Bach Mai Hospital in Hanoi. His department was virtually destroyed when the hospital was the target of the 'Christmas bombing' of the city in December 1972.

Reporting chromosomal abnormalities

The cytogenetics department is compiling a register of the incidence of malformation in several districts both in and around Hanoi. It will be the first register to provide an accurate picture in this part of Vietnam, says Tuyen. As a scientist, he is better known for his work on chromosome mapping, and as one of the authors of two papers, published in Vietnam, which claim that the incidence of chromosome damage in people exposed to the herbicide 2,4,5-T (and its dioxin contaminant) in the American defoliation programme is higher than expected. But the number of people involved is too small to indicate statistically significant trends. This is "because we did the work

during the war", says Tuyen, and he adds: "We had no opportunity to do a controlled epidemiological survey".

Some scientists in the West have levelled another criticism at Tuyen's reports. They say that the Vietnamese have reported as abnormal a level of chromosomal abnormalities—gaps and breaks—considered normal in the West. Tuyen estimates that in Vietnam these abnormalities are of the order of 0.4%; for the West and Japan, they are much higher—at 1.0% or more. To what does Tuyen attribute this higher rate? "Chemicals. Perhaps a higher background level of radiation". And is he satisfied that his laboratory techniques are above reproach? "Yes. Scientists from Holland have verified the accuracy of our work".

Lack of contact with the West

Like many of the Vietnamese scientists, Tuyen is unable to obtain much of the published literature from the West, due mainly to the effects of war, and current lack of resources. Tuyen would welcome more contact with western scientists. He feels that it is important to conduct further studies to confirm two earlier reports by American scientists on the effects of herbicides on Vietnam.

One of the reports, in the US Congressional Record for 2-9 March 1972, states that there was a marked increase in the incidence of stillbirths in one province, Tay Ninh. The second report—by Arthur Westing to the Swedish Academy of Scientists meeting in February 1977—speaks of an increase in the rate of spina bifida and cleft palate in a Saigon (Ho Chi Minh City) children's hospital from 1966-68, the years of the heaviest spraying programmes. Tuyen is convinced that the

defoliation programme has resulted in an increase in the rate of birth abnormalities and chromosomal aberrations, but, until further information is available, he acknowledges that this conclusion is still very uncertain.

The same is true of Professor Tung's suggestion that dioxin may be responsible for an increase in primary carcinoma of the liver in Vietnam. Although Tung's publications and public statements on the subject are more categorical, he says privately that dioxin cannot be identified as the sole agent; the evidence for the link with cancer is circumstantial and there is no good epidemiological evidence to support it. He feels that there are many other possible factors to be considered, with aflatoxins and viruses obvious suspects. With a department specialising in the removal of liver tumours, however, Tung has seen an increasing number of patients referred to him for surgery, who do not come from any one particular area of the country. This makes it difficult to pinpoint any one agent.

In the opinion of many western scientists, the evidence implicating dioxin is very slim. They point out that there is a remarkably short time between the deposition of dioxin—beginning in 1962—and the appearance of liver tumours. Most known carcinogens have a latency period of between 20 and 30 years.

The final group of scientists involved in the assessment of herbicide damage are the zoologists at the Vietnam Scientific Research Centre in Hanoi. Dr Ngoc Quang, one member of this group, has some preliminary evidence of changes in the pattern of bird life in sprayed areas. "Normally", he says, "there is a population of about 40 peacocks per hectare. In areas sprayed with herbicides, there are none". He also reports a reduction in the pheasant population, and adds that scientists in Vietnam are "anxious to have help to assess the damage to our fauna. The changes we have recorded may not be due to the herbicides; there may be other causes."

Resolving the 'dioxin problem' is not one of Vietnam's top priorities; there are too many other areas, medical and agricultural, requiring urgent, immediate attention. What the long-term effects will be it is impossible to say; the evidence is just not available. With contamination on this scale, Vietnam is in a unique situation not to be envied. It is to be hoped that, when she asks for assistance from the scientific world, it will be promptly forthcoming. □

Alastair Hay was recently in Vietnam as a Nature travelling fellow. He was supported in part by the London Technical Group.

The great American dream machine runs out of fuel

David Dickson discusses the dilemmas facing the Carter administration over the future shape of the US space effort

FIRST the good news. President Carter's administration has proposed that the space science budget of the National Aeronautics and Space Administration (NASA) be increased by 26.7% to a record level of \$513 million—out of a total NASA budget of \$3,305 million—in 1979.

Now the bad. There are no plans to start any new planetary missions in 1979, and it now appears that apart from Project Galileo, the Jupiter Orbiter/Probe given the go-ahead by Congress last year, no major new planetary programme is likely to be under way until the mid-1980s.

It is almost exactly 20 years since the US entered the space race with the launch of the Explorer satellite from Cape Canaveral on 30 January, 1958 as a hastily arranged response to the trauma of being beaten into space by the Russian Sputnik. It is also the 20th anniversary of the National Aeronautics and Space Act of 1958, which established NASA. And last week, in celebration of the latter event, the Senate Committee on Commerce, Science and Transportation held a symposium on the future of space science and space technology.

If the future facing space science 20 years ago lay full of hope and promise, the symposium indicated how confused and uncertain the picture is today. This confusion is reflected in doubts about the future directions that NASA should pursue, indeed doubts about the whole shape of the US space effort, including space science.

Two areas are doing well. The first is space astronomy, with exciting results on X-ray and gamma-ray emission already coming from the High Energy Astronomical Observatory (HEAO), launched last year, and more promised from the Large Space Telescope, which is being started in the 1978 budget. The second area which is also expanding, and which reflects the administration's desire to see an emphasis placed on the basic research necessary to support applied research, lies in the area of space applications. Here the relative complexity of some new applications of satellite technology, such as earth resources reconnaissance and climatology, at least in comparison to earlier applications such as telecommunications, have given rise to a significant increase in the supporting science.

But there are two areas in which current frustrations are running high, and short-term expectations—in spite of vigorous pressure—remain low. The first is in what has been called "space industrialisation", where the imagination of space engineers, with plans for mining asteroids for important minerals and creating vast solar receptors to beam energy down to earth, have so far not been matched by any major commitment from NASA. The agency claims that it is holding back work on large space structures until it sees the outcome of the Space Shuttle programme.

Planetary science

Planetary science is the second area in which NASA appears to be holding back from any major future commitment. Admittedly the present situation for planetary science is not too bad, largely because of the success of scientists in getting the Jupiter Orbiter/Probe into the 1978 budget. (Indeed, the main reason for the substantial 26.7% increase in the 1979 space science budget is last year's acceptance by Congress of this and the space telescope, both projects requiring substantially more funding in the second rather than the first year).

But the Jupiter project only survived in NASA's programme after a considerable battle in Congress, which required mustering the full letter-writing potential of the planetary science community. Although accepted by the Senate, the project was rejected in a surprise move by the House Appropriations Committee under the chairmanship of Mr Edward Boland (Dem-Mass), and only scraped through following compromises made at the conference stage.

Less fortunate has been a proposed project to send a spacecraft into a polar orbit around the Moon, making use of a set of matched sensors that would have provided a survey of the whole planet from a height of 100 km. This had been proposed within NASA in three successive years, and twice (for 1978 and 1979) put forward by NASA to the Office of Management and Budget, only to be rejected on each occasion. It now seems unlikely that NASA will be prepared to put forward this project again, preferring to rethink how it should reapproach the

Moon at a later date, possibly in five or ten years' time.

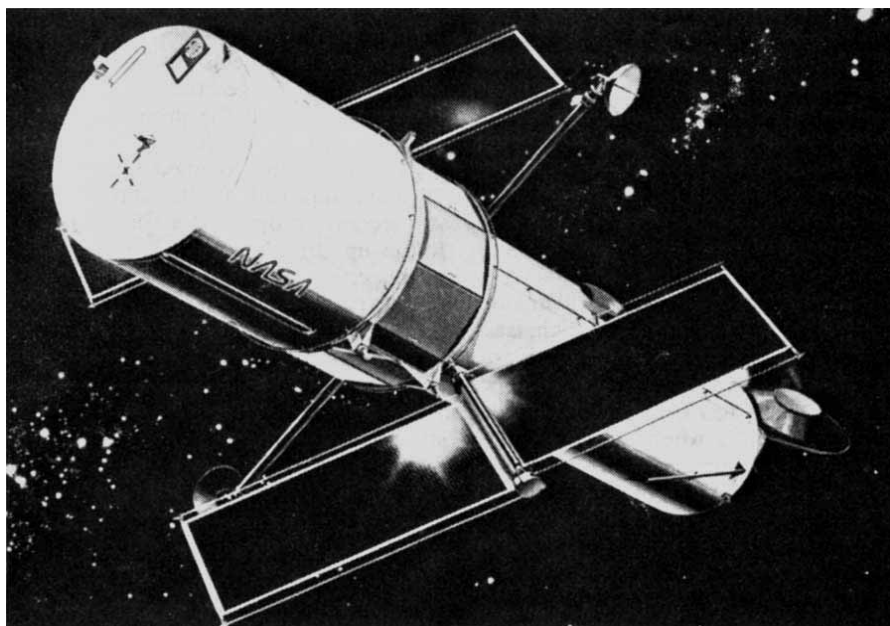
Two other projects discussed within NASA failed to get the agency's support. The first was the proposal to send a spacecraft to rendezvous with Halley's Comet, due to make its once-in-75-years approach to the sun in 1986. A second project was a proposed follow-up to the Viking mission to Mars, either sending a roving vehicle to the planet to gather more information about its surface, or to arrange for a sample of Mars rock to be brought back to earth for analysis.

The possibility of a Mars project is still receiving enthusiastic backing from scientists, particularly those interested in investigating the distribution of organic matter in the universe. And there will be a considerable push this year to get a trip to Mars in 1986—the next date when the planet will be in a suitable position relative to the Earth—into the 1980 budget.

However, it will have to compete with another equally attractive proposal, that to send an imaging radar device to orbit Venus in order to make the first accurate measurements of the planet's surface through its dense clouds. A Venus project could be particularly attractive for two reasons. Firstly, there are already two planned Pioneer trips to Venus planned for launching in May and August of this year, and interest in the planet is therefore likely to be high. Secondly, and perhaps politically more important, is the fact that the Russians are planning both a fly-by mission, probably with an orbiter, and also a joint mission with the French that will involve dropping a balloon into the planet's atmosphere.

Apart from these there are a number of additional projects being proposed, each with its group of supporters. One is to send a mission to Titan, the largest of Saturn's moons, whose atmospheric conditions make it of particular interest to space scientists with biological interests. Those disappointed by the failure to gain support for a mission to Halley's Comet still contend that a comet mission of some kind would provide invaluable information about the origins of the universe. And there is also a project being discussed to reintroduce manned investigation in the relatively near future with a mission to one of the many small bodies, such as the nuclei of burnt-out comets, that orbit the Sun close to the Earth.

Each of these projects would be fruitful from both a scientific and a technological point of view. The main problem facing planetary scientists, however, is how to secure that extra bit of commitment from the administration necessary to obtain the vast amount of resources that each would require.



NASA

Artists' impression of NASA's space telescope

Victims of earlier success

The size of the projects, the uncertainties involved, and the long-term nature of any pay-off, make it impossible to apply with any degree of accuracy the type of cost-benefit analysis that is increasingly being required of R & D efforts. As Dr Noel Hinners, associate administrator for space science at NASA, points out, "scientists have desires, not requirements."

But in their failure so far to gain the necessary backing, the hawks of both space technology and space science are partially the victims of their own earlier success. On the one hand, the apparent ease with which the space bonanzas of the 1960s were achieved, and the technological euphoria which they generated, has obscured the type of commitment needed to secure success; recent launcher failures and technical problems with Space Shuttle engines have made both administrators and politicians more cautious in their projections of feasibility.

The second spin-off from earlier successes has been that, by putting the emphasis on the more competitive aspects of getting places first, it has been made comparably harder to argue for follow-up missions of equal, if not greater, scientific value. Both the proposed lunar and Mars projects which failed to receive funding for 1979 suffered partially from this problem.

The resulting situation was expressed at the Senate symposium by Professor James Arnold, of the Department of Chemistry at California University, La Jolla, and a leading advocate of the lunar mission. "The lunar and planetary science community currently feels a great frustration based on the paradox that we have very exciting things that we know how to do, but

budgetary restrictions on doing them," he said.

This frustration, shared with those who would like to see NASA advancing rapidly into large technological structures, finds a frequent echo in Congress from those who argue the need for the US to maintain a position of superiority both in leading the pursuit of new knowledge, and in maintaining a technological advantage over other nations (NASA's formal role is according to the National Space Act to preserve the role of the US "as a leader in aeronautical and space science and technology").

But this style of political debate, however attractive it may have appeared during the Cold War period when politicians could play on the fear of Soviet technological superiority, is out of place in the current administration. The form of hegemony that President Carter sells is moral, rather than technological (just as the success of *Star Wars* is based less, as the space hawks like to suggest, on the inherent attractions of space travel as on the apparent victory of morality over technology).

Thus, however much he is attacked for showing a lack of imagination, space bonanzas, whether scientific or technological, are not Carter's style; like other candidates for federal aid, they require pragmatic justification, in the way that NASA is putting increasing emphasis on the "useful applications" of space technology—and the science necessary to achieve it—rather than the more glamorous aspects.

Dr Robert A. Frosch, NASA's newly appointed director, is under pressure from both Congress and the aerospace industry to take a more "imaginative" approach. But he is proceeding cau-

tiously, and as a result finds himself "in the somewhat paradoxical situation that as steward of NASA I have been cast in the role of the conservative, a brand new position for both me and the agency."

Looking to the future

As the NASA budget for 1979 comes under scrutiny from Congress, there are likely to be several attempts to increase both its size and its scope. Already Representative Olin Teague, for example, has been persuaded to table a resolution pressing the investigation of space resources. And representative Ronnie G. Flippo of Alabama echoed a widely-held feeling when he told the House two weeks ago that the 1979 budget was in general "a *status quo* budget, lacking in the kind of action, boldness and innovation which should characterise scientific leadership."

But in general space is much lower on Congress' priority list than it was ten years ago. Pay-offs must not only be promised, but their feasibility demonstrated. The Space Shuttle, for example, succeeded in getting through Congress largely as a result of the economies that could be demonstrated for a re-usable space vehicle, and its potential commercial viability.

The changing environment is further reflected in the uncertainty over the future of NASA, itself partially a victim of its own success. Some claim that the agency should now shift its concern from the means to the goals of space activity, helping to build what one enthusiast calls "the new space America."

Others feel that NASA's responsibility should remain essentially exploratory and experimental, although most space scientists seem to agree that the agency should remain responsible for both the basic sciences and the investigation of its implications, since the former will only thrive if conducted in a symbiotic relationship with the latter.

At present the administration in general—and President Carter in particular—seem uncertain about the direction in which NASA should turn. A study of the national space effort conducted as a result of a presidential memorandum last year by Dr Norman Brown, Secretary of State for Defense, is said to have disappointed the administration in its conclusions.

More significant for space science will be a "public declaration" on the future shape of civil space activity, including NASA, which is currently being prepared by Dr Frank Press's Office of Science and Technology Policy. Some form of statement from OSTP is expected next month; but a renewed commitment to major programmes in space still seems a long way off. □

Fermilab director resigns over funding

IN an effort to stabilise the funding of high energy physics (HEP), the US Department of Energy has decided to recommend that the HEP budget should be kept at a constant value of \$300 million (in 1979 prices) over the next few years.

According to the department, this sum—which breaks down into \$200 million for operating costs, \$40 million for equipment, and \$60 million for construction—will guarantee an active HEP programme based on three national centres, the Brookhaven National Laboratory on Long Island, the Fermi National Accelerator Laboratory in Illinois, and the Stanford Linear Accelerator Center (SLAC) in California.

However, although the department's proposed commitment is considerably higher than two years ago, when the HEP budget dropped to about \$240 million after reaching a peak of about \$500 million (both in 1979 prices) in 1970, it is still less than many scientists would like. And in protest at the department's decision not to speed up the construction of the energy doubler at Fermilab, Dr Robert Wilson, director of the laboratory, has carried out a threat made two months ago and submitted his resignation.

The Department of Energy's decision to keep HEP funding constant at a level slightly higher than the \$290 million proposed for fiscal year 1979 was taken after long and detailed discussion with the high energy physics community, in particular over the construction of the 400 GeV proton-proton colliding beam facility (ISABELLE) at Brookhaven which the department has proposed should start in 1979.

"Before agreeing to support ISABELLE, I asked what would make sense in terms of a long-term high energy physics programme?", Dr John Deutch, director of the department's Office of Energy Research, said last week. "I was not prepared to support the construction of a major new facility unless it was in the context of a balanced and aggressive high energy physics programme." Dr Deutch said that the decision to maintain a constant budget in real terms over the next few years was consistent with the consensus of the high energy physics community over the type of support that was required, and that it would provide some stability for future planning. A similar scheme was also being developed for funding nuclear physics.

The decision was welcomed by Dr Sidney Drell, deputy director and chairman of the department's High Energy Physics Advisory Panel (HEPAP), who pointed out that ever since the Ramsey report of the early

1960s, physicists have been asking for a stable basis for long-range planning. "Stable funding expectations will be very useful to us in terms of allocating resources, particularly in view of the fact that major HEP instruments can take seven years or so to build", he said. He added that the general level of funding was only "the minimum viable level" compatible with a three-centre programme, and there would still be many opportunities lost for good physics. According to Dr Drell, the proposed funding levels represent a 25% drop in operating funds for HEP facilities from their peak at the end of the 1960s.

Dr Wilson's decision to resign as director of Fermilab is a result of his failure to persuade the Department of Energy to bring forward the construction of the energy doubler, which by adding a second ring of superconducting magnets will eventually enable Fermilab both to save power costs and to reach energies of 1,000 GeV or more. Although the department is proposing to allocate \$10 million towards construction of the doubler in the fiscal year 1979, Dr Wilson had asked for

\$35 million, which would have been sufficient to complete the project during the year.

In his letter of resignation to the Universities Research Association, which runs Fermilab, Dr Wilson said that current restrictions of funding meant that the laboratory was operating at about half its potential capacity, a predicament that was particularly serious in view of the relatively high funding enjoyed by the European Centre for Nuclear Research (CERN) in Geneva, which recently brought its own 400 GeV accelerator into successful operation.

"Our scheme to leapfrog CERN's financial advantage by increasing the Fermilab proton energy to 1,000 GeV through the application of superconductivity has been confounded by indecisive and subminimal support," Dr Wilson said. He added that he had felt he was unable to continue to give the impression that he could responsibly direct Fermilab without a substantial increase in funding.

Although Dr Wilson is resigning as director, he has said that he intends to remain at Fermilab to work on the energy doubler project.

David Dickson

Salyut-6 used for weightlessness experiments

THE long sojourn of cosmonauts Yuri Romanenko and Georgii Grechko aboard Salyut-6 represents far more than an attempt to recapture the space endurance record. Soviet space planning has always been based on the idea of permanent orbital stations, indeed, Tsiolkovskii, "the father of Soviet cosmonautics" envisaged such stations as a *sine qua non* to manned lunar or interplanetary flight. The rumours which followed the Soyuz-11 disaster, which attributed the cosmonaut's deaths to the effect of prolonged weightlessness, were traumatic to space planners and amateur enthusiasts alike, and although subsequent investigations proved that the tragedy was in fact caused by a defective seal and consequent depressurisation, research into the physiology of weightlessness is a major concern to the Soviet manned space programme.

Unfortunately, weightlessness research is something which must largely be carried out *in situ*, and there is little scope for ground-level simulation. A 'hydro-weightlessness' tank is used to train cosmonauts for the difficult mechanical operations of station maintenance. Poland, as part of its contribution to the *Interkosmos* project, is said to be working on the biomedical effects of weightlessness, and an interesting experiment was recently



"You did say any kind of moral support, comrade!"

completed in Leningrad where for six months 18 volunteers lay "in a very inconvenient posture, with their feet several degrees higher than their heads", able to wash, shave, eat, and read, but "only allowed to turn from side to side, without rising even a little".

That experiment, which was presumably intended to simulate space-flight demineralisation, proved, according to its scientific director, Professor Leonid Kakurin, to be a great success.

Once the volunteers resumed ordinary life, their physiology quickly returned to normal, so that, according to Kakurin "space flight is not limited to six months. The human organism has genuinely unlimited possibilities".

On board experiments, however, are the only sure way of determining the effects of weightlessness. The biological programme of Salyut-6 includes both human and non-human tests. Certain of the latter seem somewhat esoteric—as, for example the result that tadpoles hatched in space swim in spirals, while earth-hatched tadpoles swim in a more "disorderly" manner. Other Salyut-6 experiments, monitoring the total effect of space rather than weightlessness alone, include the Franco-Soviet Cytos experiments on micro-organism cell division, a genetic investigation using *Drosophila* (banned from Soviet research at the height of Lysenkoism) and the somewhat ominously named "Medusa" experiment which compares the effect of space-flight on biopolymers in and outside the station.

The main source of medical data is, however, the on-board monitoring of cosmonauts. The Salyut-6 tests have included electrocardiograms, encephalograms and rheograph and manometer investigations, used at "rest" and after exercise on the "running track" and "veloenergometer". A special self-contained microanalysis kit was used to enable the cosmonauts to take samples of each other's blood; these were returned to earth by the Soyuz-26 craft.

The possibility of returning specimens in this way has greatly extended the facilities for maintaining the cosmonauts' health and well-being. Similarly the use of unmanned supply rockets will enable any necessary medicines to be taken into orbit. Progress-1 did, in fact, carry a replacement medicine chest—since although the original one had not been broached, it was felt that certain medicaments might have grown stale and lost their efficacy.

The use of replacement crews and supply rockets, however, has far more significance to cosmonaut health than simply that of keeping the life-support systems operative and the supplies recharged. The Salyut-6 planners have laid great emphasis on the physical comfort and psychological well-being of the cosmonauts. Station noise has been reduced by siting the motors "further astern" and installing "quieter switches". A shower has been installed (a triumph of ingenious design) and semi-rigid spacesuits are used for EVA which are said to simulate gravity by directing pressure to the lower parts of the body. The on-board menu includes over 60 named brands of food.

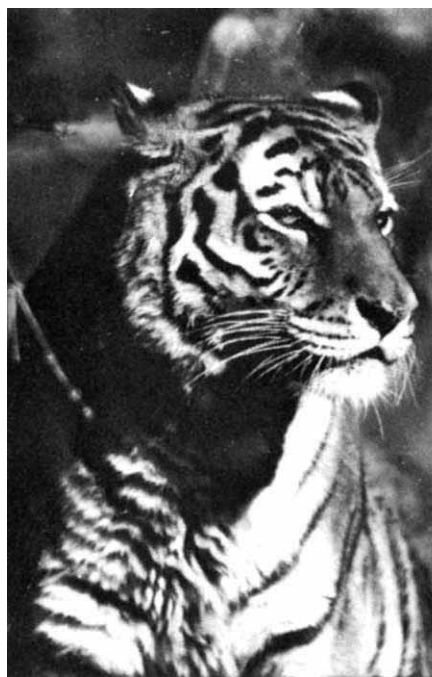
Nevertheless, all is not entirely

happy aboard Salyut-6. The TV set ferried up by Progress-1 was said to be urgently needed, to keep the crew in touch with world events. One cosmonaut mentioned that after 45 days in orbit there was a need for "any kind of moral support"; it was a great boost, he said, to know that the completed experiments returned for processing via Salyut-26 had turned out well. Clearly, one of the main problems of small-crew long-term space-stations is

likely to be that of psychological isolation—a factor which will undoubtedly increase as such stations become a matter of routine rather than headline news. One can only hope that in the future, relieving or supply crews will be less careless than Dzhaniybekov and Makarov, who in the excitement of undocking from the first four-man Salyut-link-up left behind the letters home of Romanenko and Grechko.

Vera Rich

USSR moves to protect rare animals



Vanishing species: the Amur tiger . . .



and goral

THE Soviet Union is preparing new legislation governing the conservation of rare fauna and restrictions on hunting. The December 1972 Resolution of the Central Committee and Supreme Soviet "on increasing the protection of nature and improving the use of natural resources", placed an emphasis on ecology that had been largely lacking in Soviet official thought, but so far its provisions have proved impossible to implement.

According to N. Eliseev, head of *Glavokhota* (Principal Hunting Trust) of the Russian Soviet Federative Socialist Republic (RSFSR), the poaching of rare and protected species is still rife. The RSFSR is by far the largest of the Union Republics, comprising Russia proper and the major part of Soviet Asia. Eliseev's department is thus responsible for 80% of all fur-bearing animals and 90% of all bird-life in the Soviet Union, including such rare and vanishing species as the Amur

tiger, the goral and the desman (source of musquash).

Hunting is still an important industry (since in much of the Soviet Union fur is a necessary rather than a luxury article) and it is governed by a somewhat complicated system of inspections and licences. But the poachers often manage to circumvent the various controls by making rapid safaris in fast cars and motor boats. Poaching, or at any rate, breaches of the hunting regulations on the part of licensed hunters, are common.

The new laws, says Eliseev, "will intensify the protection of wild animals, birds, fish and other fauna". Presumably this means (though he does not say so) severe penalties for poachers and dishonest hunters. Eliseev, however, stresses the positive approach to conservation, demanding a fundamental change in social attitude: "An end must be put to the consumer attitude towards nature". □

Germany's energy plans

THE West German government presented its latest programme to safeguard Germany's future energy supplies last December. An important feature of the programme concerns the efficient use of energy, especially for use in private households which accounts for the largest share of all Germany's energy consumption.

As heating makes up for 80% of private consumption, in future all new buildings will be legally required to conform to building regulations aimed at improving insulation. The government is also encouraging private owners to improve the insulation of existing buildings by offering them grants of 25% of the initial cost of improvement up to DM12,000 per purchase (£1=4DM) over the next five years. The grant also applies to the installation of solar collectors.

The new energy programme is clearly in favour of nuclear energy. Without it, the government feels that it could not guarantee an electricity supply in the long term. But just how much nuclear power station capacity is necessary is a matter of dispute particularly amongst the governing parties.

In future, however, new nuclear power stations will be sanctioned only if the problems of radioactive waste

disposal are solved. And the German view is that this can only be done by building a central plant to reprocess the spent fuel elements and to take care of the final disposal of radioactive wastes.

A site for such a plant has been chosen at Gorleben but the local population are protesting about its construction. So at the moment additional storage ponds are being built to store spent fuel elements from the power stations. The basic commitment to nuclear energy of both the federal government and the parties in the Bundestag will, therefore, only be realised if the radioactive waste disposal problem is solved.

However, the German government is also looking to coal for future energy needs. But German pit coal is very expensive and the present production of 90 million tonnes per year can be maintained only with considerable state subvention. Government and industrial research into non-nuclear energy sources is also being strengthened, the conversion of coal into other products being central to the government's programme. Research on alternative energy sources, such as solar and wind energy, is also receiving government support.

Werner Gries

Sighting the Yeti's relatives

FOLLOWING reports of a possible "Loch Ness" monster in Lake Kos Kol, Soviet scholars have postulated a possible "relation" of the Yeti. The Institute of Language, Literature, and History of the Yakut Branch of the Academy of Sciences of the USSR has collected numerous accounts of sightings of a creature known locally as the "Chuchunaa", a name which apparently is connected with the Yakut word for "fugitive" or "outcast".

According to informants, the Chuchunaa is over 2m tall, uttering deerskin, and is unable to talk, uttering only a piercing whistle. He is described as a meat-eater, and is said to have the habit of creeping up to settlements and stealing food. When the Chuchunaa sights a hunter or reindeer-herder, he usually takes flight, but on occasion, it is said, will pick a fight. (No data have been released as to the outcome of such encounters.)

The best sightings of the Chuchunaa seem to occur in summer at dawn or late dusk. Hunters, mushroom pickers and the like who are out at this time have reported seeing a wild man, with arms hanging below his knees, bare-foot and dressed in skins. The face is said to be large, human-like but very

dark, with a protruding brow "like a peak", long matted hair, and a full beard—larger than that of a man. At such times, the Chuchunaa has been reported to be feeding on berries, tearing them from the bushes with both hands.

According to Semen Nikolaev, a senior staff member of the Yakut Branch of the Academy, "almost all witnesses speak of the Chuchunaa as a reality without the fantastic detail so characteristic of legends". (To a Westerner, the above description seems very much the stuff of which legends are made—and one cannot help wondering whatever Nikolaev would classify as "fantastic"—Baba Yaga riding on her pestle, maybe?). Since the details of many sightings coincide, Nikolaev and his colleagues seem willing to admit the postulate that the Chuchunaa represents the last surviving remnant of the "Palaeoasiatic aborigines" of Siberia, who have retreated to a last refuge in the upper reaches of the Yana and Indigirka rivers. Indeed, since the last reliable sighting dates from the 1950s, some of the more pessimistic experts think that the Chuchunaa may have died out during the last two decades. □

World Bank's new health commitment

HAVING for years fought shy of involving itself in activities concerned with health, the World Bank has now taken a second step in the opposite direction—the first being its co-sponsorship of WHO/UNDP Onchocerciasis Control Programme (OCP) in the Volat River Basin. The new commitment was confirmed at a meeting at the beginning of February at WHO Headquarters in Geneva, with a 'memorandum of understanding' between the same three partners as co-sponsors of the Special Programme for Training and Research in Tropical Diseases. In general, the arrangement follows the same lines as that for the OCP, the Bank being responsible for administering the Tropical Diseases Research Fund, contributed by governments and organisations for projects to be executed by WHO and UNDP.

As expected, the cooperating parties' interests will be coordinated by a Joint Consultative Board. The board is expected to hold its first meeting in November this year. Its 30 members will include 12 representatives of governments, selected by the contributors; 12 selected by WHO's Regional Committee from among those countries directly affected by the diseases, or supporting the fund with scientific and technical assistance; three from among cooperating parties not otherwise represented; and one each from three sponsors of the programme. The latter will also have their own standing committee, meeting twice a year, concerned with technical and financial oversight of the programme.

As in the case of the OCP, there will be a scientific and technical advisory committee, concerned not only with advice on these aspects of the programme, but also with continuous independent evaluation of all programme activities. The 15 to 18 members of this committee will serve in their personal capacities, covering the whole range of biomedical and other disciplines with which the programme is concerned. The initial impetus will be maintained by interim meetings of the various groups before their formal establishment in November. A programme coordinator at the headquarters of the WHO will be responsible for the management of the programme.

As to financing, the meeting ended with some \$11 million pledged by various donors for the current year's activities. Moreover several large donors indicated that they would be making longer term pledges to cover at least the next five years of the programme.

Peter Collins

Chinese exchanges easier

CHINA has begun trying to achieve the four modernisations—agriculture, industry and defence with science and technology as the 'key'—in accordance with the policy laid out in Chairman Hua's Political Report to the Party Congress last August. The plan for agricultural modernisation over the next three years was revealed on 26 January in a report to the third national conference on agricultural mechanisation.

In spite of these moves towards achieving China's new goal, however, discussions are still taking place on the future of many aspects of national life. In education, for example, it has been reported that the advice of a top leader has been sought on a question not resolved at ministry level. It is unlikely, however, that this concerned the egalitarian drives to set up an 'open university' via the national television network and to make universal by 1985 middle-school education in the cities and junior middle-school education in rural areas. The

overall picture, however, is that progress in education is regaining momentum; an All-National Conference on Education will be held after the one on science in

China is also implementing plans to increase international academic exchange. The situation report delivered last December by Fang-Yi, Vice-President of Academia Sinica, reiterated that China is striving to learn advanced science and technology from foreign countries whilst keeping to the principle of self-reliance and independence. Already, some scientists of Chinese origin have been led to believe that they will be invited to China at some stage in the near future for short term teaching and/or research. And there is also no question that China will employ many foreign experts.

An ordinary tour of China can be simply arranged. Last month's issue of *China Reconstructs* published a guide for prospective applicants, either single or in a group. A technical visit, such as a visiting professorship, will probably

have to go through personal connections or organisations such as the revitalised Chinese Scientific and Technical Association, whose acting chairman is Chou Pei-yuan of Peking University. Chou (a physicist) may in fact be regarded as in charge of academic matters in research and education, and Fang Yi as in charge of political and general concerns.

The Japanese, however, have gone some way to taking the initiative in fostering exchange with China: an Association for Japanese-Chinese Scientific Exchange was founded in Tokyo last December. The French also recently signed a Science and Technology Agreement with China covering, amongst other cooperative schemes, the exchange of scientists. Incidentally, M Barre's state visit to China also resulted in another development: Hua's acceptance of an invitation from Giscard d'Estaing to his country. That will make France, an old friend of China's, the second country in the world that a Chinese Communist Party Chairman has ever visited.

T. B. Tang

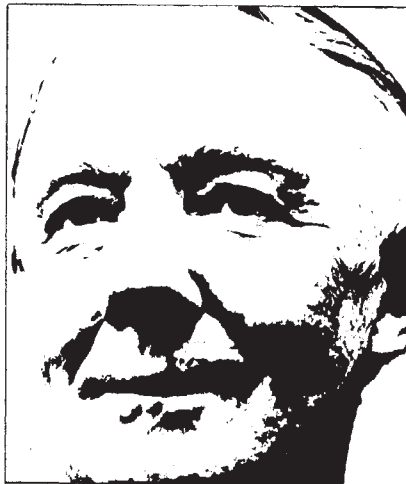
Most countries have their own units of length, weight and volume, and several systems for measuring temperature have been used in different parts of the world. These different units present difficulties to travellers, and to the communication of scientific results between nations. Many years ago, on a walking holiday in Sweden, I set off for a village which a signpost said was three miles away. After striding along at a reasonable pace for nearly two hours I saw a second sign saying that I still had to cover two miles. Thus I learned that one Swedish mile was equal to six English miles.

On another occasion at a seaside resort in Denmark I saw a notice which said that the temperature of the sea was 16 degrees. I hesitated to bathe, for this seemed a little chilly, but when I overcame my reluctance I found the water warmer than expected. The temperature had been recorded on the Réaumur scale, and corresponded to 20 degrees Celsius, or 68 degrees Fahrenheit.

It seems sensible to come to some agreement so that the same units are used internationally. For a long time scientists in Britain and many other countries have followed two systems, metric in the laboratory, and 'traditional' outside. We are now trying to change and are using the metric system everywhere. Unfortunately, this rational change makes life less interesting. It was always pleasant to find maunds and seers in India, and quite easy to make the necessary conversions. I was glad to find recently in Mauritius that land

is still measured by the arpent (1.043 acre). British farmers are urged to go metric, but they still think, and usually talk, of yields in hundredweights per acre, though the bushel has largely disappeared.

Uniform units



KENNETH MELLANBY

Those of us brought up in the laboratory on what are now old-fashioned metric units find the SI system difficult to use. I really cannot understand why I must stop talking about cubic centimetres, for this is a sensible unit and is easily understood. The change to millilitres seems to be unjustified.

Even more important than using the same units (for these can easily be con-

verted) is to have the same meaning for words. In Britain and most of Europe the billion has always been 10^{12} . North Americans, thought by Europeans to be prone to exaggeration, give the word the value of only 10^9 . I despair of converting Americans back to the correct use of the term, and see no reason to follow their bad example. I therefore believe that the term has become so ambiguous that it should no longer be used. As an editor I ban it from my journal. Recently I have been shocked to see that *Nature* has allowed the word, with its American use, to appear in its columns. I hope that all my readers will support my campaign for the total abolition of the billion in any form.

In Britain we have even decimalised our currency, and lost our useful shillings and pence. One more exotic coin, the guinea, has not yet disappeared entirely. It is still used for selling expensive race horses and to pay for private medical treatment. The guinea was originally the same as the pound sterling, and was made up of twenty shillings. However, from 1717 gold sovereigns (pounds) made of Guinea gold from West Africa were found to be so pure that they were officially valued at twenty-one shillings. I recently received a bill for dental treatment for fifty guineas; this was converted by a young clerk into £55. She apparently thought that with decimalisation the guinea was now £1.10 instead of the original £1.05. No wonder people think that decimalisation has helped to increase the rate of inflation.

correspondence

USSR bars Israeli scientist

SIR,—We wish to protest against the discriminatory practices of the Soviet Union towards Israeli scientists wishing to participate in international scientific meetings held in the USSR.

The Fourth International Meeting on Ferroelectricity (IMF-4) was held in Leningrad on 18-23 September 1977. The meeting was organised by the Academy of Sciences of the USSR under the sponsorship of the International Union of Pure and Applied Physics, the International Atomic Energy Agency, and the European Physical Society. We had both delivered papers at the Third International Meeting on Ferroelectricity in Edinburgh in 1973, and we were looking forward to participating in IMF-4. However, at every stage—soliciting information regarding the meeting, inquiring as to the status of our submitted manuscripts, attempting to obtain an entry visa to the USSR—we, and several other Israeli colleagues, encountered incredible obstacles, which ultimately led to our being unable to attend the meeting. Our individual experiences were as follows:

Sidney B. Lang submitted the abstract of a paper on 29 March 1977 to Professor G. Smolensky, Chairman of IMF-4 in Leningrad. Receipt of the abstract was never acknowledged, nor was information sent regarding the status of the paper. After seeking the assistance of Professors W. Cochran (University of Edinburgh) and W. J. Merz (R.C.A., Zurich), one of us (S.H.) received a letter from Professor Cochran, dated 30 June 1977, stating that he had received a cable from Professor Smolensky that the papers of Lang and Havlin had been accepted. Because of the impossibility of an Israeli receiving a visa to the USSR without first travelling to Western Europe, and lacking any official documentation from the Organising Committee that could be used in requesting a visa from Soviet embassies in Europe, Lang was forced to withdraw his paper on 25 August 1977. On 16 September 1977, two days before the meeting opened, Lang received the 'second circular' about the meeting, mailed from Romania on 30 August. This circular bore no date, but it specified a number of deadlines between 30 April and 18 June.

Shlomo Havlin submitted the abstract of a paper on 7 February to Pro-

fessor Smolensky and as in the case of Lang, heard nothing. On 8 July the Secretary General of IUPAP, in response to requests for assistance by the Israel Physical Society, cabled that Havlin's paper was included in the meeting programme, and that he need only apply to Intourist for an entry visa. On 9 August, Havlin arrived in Paris and immediately applied for a visa at the Intourist office. At every subsequent visit to the office as well as to the Soviet Embassy, he was assured that his visa would be issued "tomorrow". The seemingly sincere promise that the visa would surely be forthcoming led Havlin to remain in Paris beyond 8 September when he had planned leaving for Leningrad. On 11 September, still with no visa, Havlin realised there was no point in continuing his battle and left Paris.

Although the organisers of the Leningrad meeting had made firm promises to IUPAP, Professor Cochran, and Professor Merz, the Soviet authorities refused to issue a visa. From our experiences, it is obvious that Israeli attendance at this international meeting was prevented by the Soviet Union.

We suggest the following to our fellow physicists and to the officials of physics organisations:

- International organisations should not sponsor meetings in the USSR without firm guarantees from the Soviet government that these meetings will be open to citizens of all nations without exception. Promises from Soviet organising committees should not be accepted *in lieu* of official Soviet government commitments. Scientists should write to the officers of the physics organisations to which they belong, demanding that this principle be strictly adhered to.
- Scientists should refuse to attend meetings in the USSR unless they are certain that the Soviet government has, in fact, guaranteed that the meetings will be open to citizens of all nations without exception.
- Letters should be written to the Soviet organisers of IMF-4 protesting the discrimination that was practised towards Israeli scientists. The organisers were:

Chairman:

Professor G. Smolensky
A.F. Ioffe Physico-Technical Institute
Academy of Sciences of the USSR
Leningrad, 194021
USSR

Vice-Chairmen:

Professors L. Shuvalov and I. Zheludev
Institute of Crystallography
Academy of Sciences of the USSR
Moscow, 117333
USSR

We believe that the holding of meetings without firm adherence to the above principles is totally incompatible with the international character of science and scientific research.

SIDNEY B. LANG

*Ben-Gurion University of the Negev,
Beersheva, Israel*

SHLOMO HAVLIN

*Bar-Ilan University,
Ramat-Gan, Israel*

Burying high-level wastes

SIR,—The disposal of intermediate and low level radioactive wastes in salt deposits of Permian (about 220 Myr) age is proceeding and proposals have been made to treat high level wastes in a similar fashion. When evaluating the suitability of these formations for this purpose, the proponents stress the antiquity, aseismicity and availability and conclude that they are "extremely stable" (*The Management of Radioactive Wastes*, IAEA, Vienna; 1977). Most geologists, however, would consider that rocks of this type are very unstable. Widespread halokinesis is well-documented and the role of diapirism in the formation of stratigraphic traps favourable for oil and gas accumulation is particularly significant.

In rocks containing potassium the radioactive decay of ^{40}K forms radiogenic ^{40}Ar , resulting in a high relative abundance of this isotope in the atmosphere. In early experiments designed to evaluate the possibility of using this decay scheme to measure geological time the ^{40}Ar concentrations in samples of evaporite were measured. In almost all cases it was found that significant argon loss had occurred. This was attributed to the instability of these strata and their susceptibility to dissolution by fluids (such as percolating ground water) and subsequent recrystallisation which had allowed the escape of ^{40}Ar . It is not usually possible to detect these effects by examination of the rocks in hand specimen or thin section. For instance, although Suess (1948), consulted "specialists for salt mines" his analyses demonstrated that

the samples he had been given had been recrystallised in recent times (Houtermans, F. G. in *Potassium-Argon Dating*, 5 Springer-Verlag; 1966). As a result the evaporitic minerals have not subsequently been routinely employed for K-Ar dating.

The apparent ease of escape of radiogenic daughter isotopes from salt formations, particularly in recent times, and the alarming implications in the event of a containment failure, seem to require a thorough re-evaluation of the stability of these beds. Before any final decision on high-level radioactive waste disposal is reached, a systematic programme of argon measurements might provide a simple means of determining the extent of this phenomenon and enable its significance to be assessed.

R. M. MACINTYRE

University of Strathclyde, Glasgow

Alcohol in Britain

SIR,—J. A. Spring and D. H. Buss's account of three centuries of alcohol in the British diet (*Nature* 270, 567–72; 1977) gives good evidence for the high intake of beer in Britain in years gone by. But may I add a comment to the statement that "there are no obvious reasons" for the steady decline in beer consumption during the eighteenth century (p 567)? It is that the increase in tea-drinking in that period was very much greater than taxation figures suggest.

Genuine China tea remained expensive throughout, but a great deal of what was consumed was in fact adulterated with the dried and chemically treated leaves of such trees as hawthorn, sloe and ash (known in the trade as 'smouch'), or with used tealeaves purchased at small cost from the kitchen-maids of the wealthy. The cheapest teas contained little else.

Tea-drinking increased steadily during the century in all classes of society, especially among women. For the poorest families in the South and Midlands, there was a reason for this increase. Enclosure and the high cost of fuel deprived them of soups and the cheapest cuts of meat, which required long cooking. Crude tea without milk was the only warm comforting element in a diet that otherwise consisted of bread, cheese and occasionally cold, fat bacon.

The changeover from beer to tea as the common mealtime drink was well under way during the 1730s and 1740s. And in 1784 the high import duty on tea was at last lowered to a nominal sum on the grounds that "tea has become an economical substitute to the middle and lower classes of society for

malt liquor, the price of which renders it impossible for them to procure the quantity sufficient for them as their only drink".

C. ANNE WILSON

The Brotherton Library,
Leeds

Dietary lipids and heart disease

SIR,—May I point out some fallacies in Dr John Rivers' review (*Nature* 270, 2; 1977) of the so-called 'lipid hypothesis' of coronary heart disease (CHD)?

●The Finnish trial of Miettinen *et al* did show a notable decrease in mortality for males on a diet relatively high in poly-unsaturated fat. During the transcription of the mortality data from the original paper (Miettinen *et al*, *Lancet* 2, 837; 1972) to the article discussed by your reviewer (Mann, G., *N. Engl. J. Med.* 297, 646; 1977) an error was made which obscures the difference in mortality between the experimental and the control period ($32.00 + 2.84$ does not equal 38.84). It is disappointing that neither the editor of the *N. Engl. J. Med.* nor your correspondent noticed this.

●Unlike cigarette smoking, atmospheric pollution has not been shown to be an independent risk factor for CHD.

●Poly-unsaturated margarines do not necessarily contain *trans* fatty acids. The most popular Dutch brand of poly-unsaturated margarines contains 60 to 65% *cis-cis*-linoleic acid and 0% *trans* fatty acids. Its British equivalent ('Flora') has 6% of its fatty acids as *trans* isomers, which is similar to the proportion found in butter (data by courtesy of Unilever Research, Vlaardingen, The Netherlands).

●Epidemiological data overwhelmingly point in the direction of a relation between consumption of saturated fat and CHD (Keys A., *Atherosclerosis* 22, 149–192; 1975). The Masai are only a notable exception. Incidentally it should be stressed that cause-effect relationships cannot be proved or disproved by such comparisons between tropical and western populations, but only by controlled experiments.

●Few people with original or heretic ideas in the food-health field are finding it really difficult to obtain research funds or publicity. Dr Mann himself, as the recipient of a career investigatorship from the National Heart, Lung and Blood Institute, is a case in point.

Nutrition is a billion-dollar business, a favourite subject for the media and an almost religious pre-occupation for a large part of the public. In view of these pressures, scientists bear a heavy responsibility to keep their data straight. In my opinion neither Dr

Mann nor your correspondent have succeeded in this.

MARTIJN B. KATAN

The Agricultural University,
Wageningen, The Netherlands

Intelligence is more than IQ

SIR,—I was interested in the letter by Brian J. Ford (5 January, page 7). I agree very fully with him of course, that the controversy is confused by the impossibility of obtaining measurements independent of cultural background. It is a great pity that so much more ink is being spilt in trying to prove that the heredity factor is unimportant, which for all I know may be true, than in the effort to raise all cultural backgrounds to an optimum level.

I would like to amplify Mr Ford's comment on the need for producing criteria which would reconcile mental measurements with the realities of life. What is practically accepted as intelligence depends to a very much more limited extent on IQ than is usually recognised. Thus IQ measurements often depend largely on the accurate understanding of the exact meanings of words and on the solving of logical problems. Solving real practical problems may often involve no language at all and in nearly all cases is concerned with the realities of a complicated situation rather than with the exact words by which this should be described. Furthermore the problems presented are unnatural in that the examinee knows that he has all the data required and that every datum is correct.

In real-life problems one never knows in advance whether one has all the necessary data and often is unsure whether the available data are right. Practical intelligence is therefore concerned with the judgement of whether sufficient data are available, with an understanding of how to obtain fresh data when necessary and then assessing the correctness or otherwise of the vital parts of the data available. This will involve persistence—in few practical situations must one solve 12 problems in 36 minutes—an ability to avoid prejudice, and some capacity for lateral thinking as well as IQ. Having satisfactorily solved all the practical problems the logical problem is nearly always trivial.

While a high IQ is valuable in a number of ways and probably has some correlation with the other factors I have mentioned, it is most unfortunate that it is so widely regarded as more important than it is, merely because it is so easy to put it in numerical terms.

J. H. FREMLIN

The University of Birmingham, UK

news and views

H-2 and HLA antigen structure

from Jonathan Howard

THERE have recently been remarkable developments in understanding the structure of the antigens of the major histocompatibility complex (MHC). For a variety of reasons the structures of these molecules are of consuming interest. First, they are powerful antigens; T cell responses to MHC antigens are one or more orders of magnitude stronger than other antigens. So abundant are T cells reacting to MHC antigens that it is at least debatable that the entire T cell pool is committed to the recognition of these structures.

Serologically the MHC antigens are exceedingly complex; each molecule, particularly those specified by the human HLA-A and HLA-B genes, and their mouse homologues H2-K and H2-D, carries multiple antigenic determinants which constitute in sum the families of specificities that identify each MHC product. This serological complexity implies a genetic polymorphism of unprecedented extent. The presence of multiple alloantigenic specificities on a single molecule probably means that there is not just a single highly polymorphic site but several independently polymorphic regions on each molecule. Comparative sequence analysis of H-2 and HLA molecules available for the past couple of years (see *News and Views* 261, 189; 1976) suggests that the antigenic differences between H-2 and HLA allotypes reflect multiple amino acid substitutions at least at the NH₂-terminus, and tryptic peptide analysis of whole H-2 molecules shows that amino acid variation between allotypes must extend throughout a large portion of the molecule (Brown *et al. Biochemistry* 13, 3174; 1974). The origin and maintenance of such complex allotypes is a genetical problem of considerable interest in its own right (Silver & Hood in *Cont. Topics molec. Immun.* 5, 35; 1976).

MHC structures apparently corresponding to those identified as polymorphic antigens are also involved in the recognition by T cells of conventional

foreign antigens (Munro & Bright *Nature* 204, 145; 1976; Doherty *et al. Transplant. Rev.* 29, 89; 1976). Recognition of self MHC structures in either a native or altered form is essential for the induction and expression of specific T cell function to other antigens: this phenomenon of 'MHC restriction' implies either that T cells carry separate receptors specific for 'self' MHC structures and for foreign antigen (the 'dual recognition' hypothesis), or alternatively, foreign antigen is captured on the cell surface and presented to reactive T cells in some form of molecular association with self MHC structures (the 'altered-self' hypothesis).

It has been known for some time that the HLA-A and B molecules, and H-2K and H-2D molecules are physically associated with β_2 -microglobulin, a polypeptide with a molecular weight of 12,500 which in both sequence and intrachain disulphide bridge structure is homologous with a single domain of immunoglobulin. This has provoked speculation that the antigenic polypeptide chain of H-2 and HLA molecules might itself have sequence or structural homology with immunoglobulin, although this was not convincingly demonstrated by the first NH₂-terminal sequences that became available.

Detailed structural studies, primarily on the HLA-B7 molecule, by Strominger and colleagues (Strominger *et al. Proc. 3rd Int. Cong. Immun.* 1977, in the press) have given extraordinary new insights into the molecular organisation of a major transplantation antigen. Most striking of all is the unambiguous demonstration that this molecule does indeed have significant sequence and structural homology with immunoglobulin. This leaves little doubt that, as has been suggested before, the two molecular systems have had an, albeit distant, common precursor. The association of HLA with the Ig-domain-like β_2 M polypeptide may therefore legitimately be considered in the same context as the association of the evolutionarily divergent but homologous light and heavy chains of immunoglobulin.

The HLA-A and B molecules are

typical transmembrane glycoproteins with a molecular weight of about 45,000 having a strongly hydrophilic C-terminal intracellular segment, a short hydrophobic segment presumably responsible for membrane insertion, and a long extracellular hydrophilic segment representing about 80% of the molecule. The extracellular segment is folded into two non-intercalated disulphide-bridged loops and a free N-terminal segment. Each intrachain loop encloses a segment of approximately the same length as the intrachain loops of Ig domains. Sequence determinations near both the disulphide bridges reveal marked homology with immunoglobulin V_H sequence around the disulphide bridge at residue 95. Furthermore the N-terminal sequence of HLA-A and HLA-B, although not itself looped by a disulphide bridge, shows a significant homology with the same V_H region after appropriate alignment.

It is difficult to escape the conclusion that the extracellular portion of HLA is composed of at least two and possibly three mutually homologous domains, all showing significant homology with immunoglobulin, and two of them looped by disulphide bridges as in Ig. A peculiar aspect of the sequence homology between HLA and the V_H of Ig is that the relevant sequence runs from the C-terminal Cys of the first intrachain disulphide bridge toward the N-terminus in the V_H, and is thus entirely included in the looped segment; in HLA the homologous sequences run from the N-terminal Cys of the two intrachain bridges towards the N-terminus, and are thus entirely excluded from the looped segments. This important difference may well indicate that the similarity in domain structure between the two classes of molecule is more apparent than real: nevertheless the overall appearance remains of two, however distantly, related molecular species.

Although the residues responsible for the serologically detectable alloantigenic activity of H-2 and HLA molecules have not been identified, it is tempting to relate the extensive sequence variation throughout the molecules to the newly

Jonathan Howard is at the Institute of Animal Physiology, Babraham.

recognised domain structure and argue that the alloantigenic complexity of the molecules is due to independent sequence variation in each domain. In this way it is possible to have unique antigenic specificities (private antigens) associated in different molecules with various clusters of other specificities (public antigens). It may be anticipated that sequence variation associated with serological activity will be found to be spread over many parts of the molecule.

It seems unlikely, however, that alloantigenicity is the most significant aspect of H-2 and HLA molecules for all its genetical and clinical importance. The recent discovery of H-2 and HLA restriction suggests that polymorphic structures on these molecules play an important part in T cell induction by antigen in the normal animal. Of particular interest therefore, is the relationship between the structure of the molecules involved in restricted interactions and the process of restriction itself. The mouse H-2K^b mutant H-2K^{ba} is a variant of considerable importance because, with respect to H-2 restricted lysis of virus-infected cells the mutant H-2 molecule is essentially non-overlapping with the wild type. T killer cells raised against virus-infected cells in the wild type H-2K^b mouse will lyse appropriately infected H-2K^b targets but not infected H-2K^{ba} targets, and *vice versa*. Comparison of the structure of H-2K^b and H-2K^{ba} molecules by Nathenson and

colleagues (*Cold Spring Harbour Symp. quant. Biol.* **41**, 343; 1977) has shown a limited number of peptide differences, compatible with a single amino acid substitution.

Furthermore, preliminary alignment of the variant peptides in the tentative model for H-2K (which is less developed than that of HLA-B7) suggests that they lie in or near the first disulphide-bridged domain. A location partially overlapping with H-2^{ba} is suggested for the non-identical but similar variant H-2K^{bd}. These findings indicate that the specificity of H-2 restriction is determined by a relatively limited part of the H-2K molecule. Interestingly, the variants H-2K^{ba} and H-2K^{bd} are not yet distinguishable from H-2K^b by conventional serological techniques, yet both variants provoke the full range of T cell responses by the parental type. It seems, therefore, that the mutated sites in these two variants are different from the predominant antigenic sites recognised by alloantibody.

Such observations have important implications for altered self or H-2 modification theories. The fact that the differences between H-2^{ba} and H-2^{bd} lie in a specific segment of the H-2 molecule implies that this segment must be important in the process of structural modification of H-2 antigen. Since the principal obstacle to the acceptance of an H-2 modification theory of H-2 restriction is the lack of any molecular mechanism by which antigen-specific modification might

occur, the localisation of a restriction site in a molecule whose structural organisation is beginning to be known should obviously be taken very seriously.

For the time being, the domain structure, association with β_2 -microglobulin and Ig sequence homology of H-2 and HLA seem significant without carrying a clear message. Without evidence for clonal allocation of different H-2 or HLA molecules between lymphocyte subpopulations, there are no grounds for believing that the specificity of antigen recognition by lymphocytes is due to sequence variations in these molecules. Nevertheless the apparent similarity between the two molecules may suggest that H-2 and HLA molecules are receptors for something at the cell surface. An analogy might be drawn with the two polypeptide hormones insulin and relaxin which, markedly similar in conformation, and plainly homologous, have entirely different biological roles while both function as hormones. I find it tempting to consider the possibility that the structure of H-2 and HLA molecules will indeed prove to be related to the phenomenon of MHC restriction by structural modification. However that may be, it is clear that the primary functions of these extraordinary molecules must be sought in the membranes into which they are inserted, rather than in their remarkable but probably biologically irrelevant immunogenicity in allogeneic responses. □

Invertible DNA in phage Mu

from Martha M. Howe

INVERTIBLE DNA sequences in prokaryotic DNA were first detected in the early 1970s during analysis of DNA from bacteriophage Mu. Since then, the discovery that inversion of a DNA segment controls the phase variation of flagellar antigens in *Salmonella* (Zieg *et al.* *Science* **196**, 170; 1977) and that inversion of an insertion sequence affects expression of the *gal* operon in *Escherichia coli* (Saedler *et al.* *Mol. gen. Genet.* **132**, 265; 1974), have lent credence to the hypothesis that inversion of DNA segments may be a general mechanism for the control of gene expression. Only recently has the role of DNA segment inversion in Mu development and gene expression begun to be understood.

An invertible DNA sequence in Mu was discovered in several laboratories studying the DNA forms arising after denaturation and reannealing of Mu DNA extracted from the mature phage

particle (Bade *J. Virol.* **10**, 1205; 1972; Daniell *et al.* *Virology* **51**, 237; 1973; Waggoner *et al.* *Proc. natn. Acad. Sci. U.S.A.* **71**, 1255; 1974). The two structures observed are shown in Fig. 1. One form was completely double stranded except for non-hybridizing single strands—'split ends'—found at the right end of the molecule. These split ends contain host DNA sequences which differ in different phage particles (Daniell *et al.* *J. Virol.* **15**, 237; 1975). The second form contained not only the split ends but also a single-stranded bubble structure termed the G bubble, flanked on its left and right by duplex DNA, the α and β segments, respectively. Hsu and Davidson (*Virology* **58**, 229; 1974) demonstrated that this G bubble arose by reannealing of Mu DNA strands containing the G segment DNA in opposite orientations. They postulated that inversion of G segment DNA occurred by recombination between two short inverted repeat sequences located at its ends and that the inversion was catalysed by a phage-specified function

active in both prophage and vegetative states.

One puzzling observation made by Daniell *et al.* (*Proc. natn. Acad. Sci. U.S.A.* **70**, 2153; 1973) was that the relative amounts of the two orientations differed in different phage populations. In DNA from lysates grown by induction of a lysogen both orientations of the G segment were present in approximately equal amounts; however, in lysates grown by infection 99% of the DNA had the G segment in one orientation, and this orientation was the same in all DNAs from lysates. The reason for this difference was not understood until experiments of Chow *et al.* (*J. molec. Biol.* **113**, 591; 1977) suggested that phage containing the G segment in opposite orientations might differ in their viability. This hypothesis was based on analysis of two classes of viable Mu phage containing partial deletions of the G and β DNA segments. Phage in one class were deleted for part of both the G and β segments and were unable to invert the G segment, pre-

Martha M. Howe is Associate Professor in the Department of Bacteriology at the University of Wisconsin, Madison.

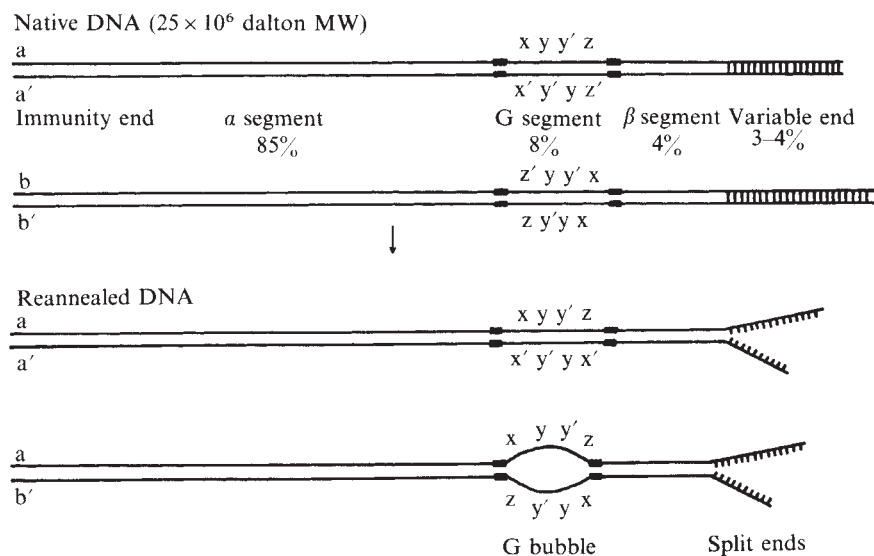


Fig. 1 Structure of Mu DNA. Structures observed in electron micrographs of denatured and reannealed Mu DNA. The $xyy'z$ and complementary $x'y'yz'$ lettering in the G segment does not represent specific genes (Hsu & Davidson *Virology* 58, 229; 1974). The black boxes flanking the G segment represent the inverted repeat sequences which serve as the sites of recombination for inversion.

sumably due to loss of one of the inverted repeat sequences, demonstrating that the process of inversion of the G segment itself was not essential for phage viability. Phage in the second class had an intact G segment and were only deleted for part of the β segment, yet they were also unable to invert the G segment. Chow *et al.* (*op. cit.*) postulated that phage in the second class were defective in the Mu-specific function needed to invert the G segment, which they termed the *gin* (G inversion) function. It was the unexpected finding that both classes of phage contained the G segment in the same orientation as that predominating in lysates grown by infection that led to the hypothesis that phage containing the G segment in the opposite orientation might be non-viable due to lack of expression of essential genes located within the G segment DNA. Further experiments in their own and other laboratories have proved the validity of these hypotheses.

Symonds and Coelho (*Nature*, 271, 573; 1978) find that in single burst experiments with non-deleted heat-inducible Mu lysogens only half the cells released viable plaque-forming phage on induction. This is expected if half the cells contained Mu prophages with the G segment in the non-viable orientation. Kamp *et al.* (*Nature*, 271, 577; 1978) confirm and extend this finding by testing populations of cells lysogenic for a heat-inducible Mu prophage for their relative ability to release plaque-forming phage on induction and for the orientation of the G segment in DNA extracted from phage particles produced as a result of induction. They found that the proportion of cells able to release plaque-forming phage was directly correlated with the proportion of phage DNA containing the G segment in the viable orientation. In a second experiment they demonstrated that

a Mu *gin*⁻ prophage could be complemented for inversion of the G segment by transient exposure of the cell to a Mu *gin*⁺ prophage. Two types of Mu *gin*⁻ lysogens resulted. The first released plaque-forming phage particles whose G segment was in the original viable orientation. The second released phage particles which could not form plaques and whose DNA contained the G segment in the opposite or non-viable orientation. Thus they have definitively demonstrated that the orientation of the G segment affects the viability of the phage particle.

Experiments by Bukhari and Ambrosio (*Nature*, 271, 575; 1978) reveal that the phage with the G segment in the wrong orientation are non-viable because they cannot adsorb to cells, whereas phage with the G segment in the viable orientation adsorbed to cells and replicated their DNA normally.

Mu genes whose expression might be determined by the orientation of the G segment have been identified in our laboratory. We have found that the essential genes *S* and *U* of Mu map within the invertible G segment. Although the functions of these genes have not been conclusively determined, several lines of evidence suggest that they may be involved in adsorption. First, serum blocking experiments indicated that the phage inactivating activity in anti-Mu serum was directed against the *S* protein of the phage particle. Second, Mu *S*⁻ and *U*⁻ phage which acquired an *S*⁺ or *U*⁺ phenotype by recombination with phage P1 often simultaneously acquired the host range of P1 (Toussaint *et al.*, private communication). These recombinants probably arose through exchange in the regions containing the G segment of Mu and a similar invertible segment of P1 which has extensive homo-

logy to the Mu G segment (Chow & Bukhari *Virology* 74, 242; 1976) and which may determine the host range properties of P1 (Toussaint *et al. op. cit.*).

The experiments described above indicate that the orientation of the G segment of Mu determines the expression of adsorption functions of Mu, perhaps by controlling the synthesis of the *S* and *U* proteins. Why should such relatively unrelated phages as Mu and P1 have homologous invertible segments and why should Mu have an invertible segment whose inversion makes the phage particle non-viable? Possible explanations were discussed at the Bacteriophage Meetings held at Cold Spring Harbor Laboratory last August. Normal genetic exchange by homologous recombination between ancestors of Mu and P1 might explain the invertible segment homology in the two phages; however, the lack of homology elsewhere in the phage suggests the intriguing possibility that the invertible segments may be able to translocate independently from one DNA to another. In this case Mu and P1 might have acquired the segment from a common source, or the segment present on one of the phages might have translocated to the other. The different lengths of the inverted repeat sequences of the elements in two phages in the translocated element suggests that changes may have occurred subsequently (Chow & Bukhari *Virology* 74, 242; 1976).

Why does Mu possess a function which impairs its viability. It may be pertinent to consider the organisation of the control system. Two possible simple on-off control mechanisms might exist. One is that postulated for the control of antigenic phase variation in *Salmonella* (Zieg *et al. Science* 196, 170; 1977) in which the promoter is present on the invertible sequence and the structural gene under its control is not. The second, perhaps more applicable to Mu G segment inversion, is one in which the structural genes are located in the invertible segment with the promoter outside. In both these cases the synthesis of the structural gene products would depend on the orientation of the invertible segment. A third possible organization is one in which the invertible segment may contain genes at each end which specify different adsorption proteins responsible for determining the host range of the phage. If the promoter for these genes were located outside the invertible segment such that only the genes immediately adjacent to the promoter were expressed, inversion of the segment might lead to expression of a different host range. The ability of the phage to alternate between two different host ranges might provide it with a selective advantage in natural populations. Such a model might be directly applicable to P1 since P1 DNA contains equal amounts of both orientations regardless of its mode of growth (Ohtsubo,

cited in Chow & Bukhari *Virology* **74**, 242; 1976). To apply the model to Mu it would only be necessary to postulate either that Mu might be defective in the second set of genes or that Mu phage with the G segment in the non-viable orientation are able to adsorb to other

bacterial strains which have not yet been tested. Further experiments will more completely elucidate the role of G segment inversion in expression of Mu genes and will reveal whether inversion of DNA segments comprises a general mechanism of gene control. □

How plants respond to stress

from Hamlyn G. Jones

THE plant hormone abscisic acid (ABA) has been invoked to explain a host of plant processes ranging from dormancy and leaf abscission, through flowering and fruit drop to the response of roots to gravity. However, it is as a stress hormone involved in the drought-induced closure of stomata, that there is the most complete evidence for a natural role for this hormone. The stomata are the chief control on water loss from plants, so that stomatal closure is an important mechanism enabling plants to survive in dry environments.

There is now evidence that the amount of ABA in tissues of many plant species increases rapidly when the plants are subjected to various stresses, including water deficits. Increased concentrations of ABA can be detected within 7 minutes of the start of a stress period and can increase as much as 40-fold over 4 hours. The evidence that this stress-induced ABA is involved in at least some of the natural stomatal movements is now overwhelming. Not only is the amount of ABA extractable from leaves generally related to stomatal aperture, but ABA applied to leaves usually results in rapid stomatal closure. Further evidence comes from work by Tal and coworkers at the University of the Negev on a wilted mutant of tomato (*flacca*) which does not close its stomata. This lesion was found to be due to a deficiency of ABA which could be overcome by supplying extra hormone.

Two papers in this issue of *Nature* (Itai *et al.*, page 652 and Itai & Meidner, page 653), provide further evidence of a role for ABA in stomatal physiology. These workers demonstrate, using microautoradiography of leaf or epidermal tissue supplied with ^{14}C -labelled ABA, that the hormone can accumulate at the presumed site of action—the stomatal apparatus.

This extends recent work by Loveys (*Physiol. Plant.* **40**, 6; 1977) who showed that although ABA does not appear to be synthesised in the epidermis it does accumulate in the epidermis

under stress. Nevertheless, there has not yet been an unequivocal demonstration that ABA accumulates in the stomatal apparatus during natural stress.

If the details of ABA accumulation are not yet proven, the mode of action of the hormone is even less certain. Changes in stomatal aperture are directly caused by changes in the balance between the internal pressure of the guard cells (specialised cells which surround the stomatal pore) and other epidermal cells. The high pressures necessary to open stomata arise from the active accumulation of osmotically active molecules and ions, especially K^+ . It is likely that ABA acts in some undetermined manner on the accumulation or retention of K^+ , as discussed by Itai and Meidner.

In spite of these recent successes in explaining stomatal movements in terms of ABA it is as well to remember that this is not the only mechanism involved in controlling stomatal movements. In the first place, so-called hydroactive closure can begin within a few seconds of changes of leaf water status; a response which is too rapid to involve a hormone such as ABA. Second, the hormone is probably not involved in the normal diurnal opening and closing movements found in most plants. Third, there are several reports of the involvement of other naturally occurring compounds in the regulation of stomatal aperture, particularly under stress. Most evidence has been adduced for an involvement of all-*trans*-farnesol (a sesquiterpenoid like ABA) which Mansfield's group at Lancaster have reported (Ogunkanmi *et al. Planta* **117**, 293; 1974) to accumulate in drought-stressed sorghum and to be effective at closing stomata.

The question still remains as to whether the postulated role for ABA in promoting stomatal closure is the main role for the apparently excessive quantities of the hormone produced in stress conditions. Many of the known effects of ABA, including reduction in leaf growth or even enhanced leaf senescence (see Milborrow *A. Rev. Pl. Physiol.* **25**, 259; 1974) as well as the reduced proportion of epidermal cells which form stomata and the increased

production of epidermal hairs (Quarrie & Jones *J. exp. Bot.* **28**, 192; 1977), are also well known symptoms of water stress. In the long term, these changes, particularly the reduction in leaf area, may be as important as stomatal closure in reducing water use by plants. Other work (Glinka *Pl. Physiol.* **51**, 217; 1973) indicates that ABA can increase the rate of water uptake by plant roots. Taken together, these results suggest that ABA may be involved in a whole syndrome of responses, all of which may contribute to adaptation to stress environments.

Perhaps many of the other reported effects of ABA, such as those on flowering, may also prove to be adaptations to stress environments. Unfortunately much of the evidence on the developmental effects of ABA is contradictory, so there is a clear need for more careful studies of the effects of applied ABA, taking account of the quantities of the hormone getting into the tissue, its distribution and its ultimate fate.

Research on ABA may have important practical consequences. Following the demonstration that some drought-tolerant species or varieties produce more ABA than sensitive ones (Larqué-Saavedra & Wain *Nature* **251**, 716; 1974; *Ann. appl. biol.* **83**, 291; 1976), several groups around the world are currently screening agricultural crops for ABA production in attempts to breed new varieties capable of high yields in dry regions. Other work concentrates on the use of ABA or its analogues as anti-transpirants to be applied to crops when drought occurs. Much of this work involves the implicit assumption that stomatal closure due to enhanced ABA levels is advantageous in dry situations. Unfortunately the reduction in photosynthesis and harvestable yield which accompanies stomatal closure may outweigh any saving in water, while rapid stomatal closure does not always appear to improve drought tolerance in crops. □

The man-made mouse

from John K. Heath

TERATOCARCINOMAS have been the subject of intensive research effort over the past few years. They are malignant tumours composed of 'undifferentiated' embryonal carcinoma stem cells (EC cells) which can differentiate *in vivo* or *in vitro* into apparently normal differentiated tissue. Since the cells can grow and

John K. Heath is in the Department of Zoology, University of Oxford.

Hamlyn G. Jones is a Lecturer in the Department of Botany, University of Glasgow.

differentiate in culture they could provide a large scale source of material whose behaviour mimics that of the relatively inaccessible mammalian embryo.

The teratocarcinoma story took on a new perspective when it was established by Brinster (*J. exp. Med.* **140**, 1049; 1970), Illmensee and Mintz (*Proc. natn. Acad. Sci. U.S.A.* **72**, 3585; **73**, 549; 1975) and Papaioannou *et al.* (*Nature* **258**, 70; 1975) that when an EC cell from a teratocarcinoma was injected into a normal mouse blastocyst, it could differentiate into fully functional, non-malignant, normal mouse tissue. The possibilities opened up by these observations are enormous. One can suppress the malignancy of an EC cell by placing it in a normal developmental environment. It might be possible to select mutations in culture which are not currently available in normal mouse stocks and examine their effect *in vivo*, thus generating mouse models of human genetic disorders. The ultimate goal would be to achieve a form of genetic engineering at the whole organism level by incorporating genetically manipulated EC cell derivatives into the germ line of a normal mouse and so produce mutant mouse stocks created *in vitro*; the man-made mouse.

Two important advances towards this goal have been achieved. Papaioannou and her colleagues (*Nature*, *op. cit.*; *J. exp. embryol. Morph.* in the press) have shown that EC cells from some cultured teratocarcinoma cell lines can also form functional differentiated tissue in a mouse injected with the cells at the blastocyst stage. This observation has been extended by Dewey *et al.* (*Proc. natn. Acad. Sci. U.S.A.* **74**, 5564; 1977) who have been able to produce a number of mice containing tissues derived from mutant injected cells. The EC cells in question lacked the X-linked enzyme hypoxanthine phosphoribosyl transferase (HPRT). This is a good choice of mutation because it is not available in mouse stocks, but can be readily obtained in cultured cells. HPRT deficiency produces a severe neurological disorder, Lesch-Nyhan syndrome, in humans. A deficiency of this enzyme in humans can also lead to some types of gout. Ten of the mice which have been analysed so far in this study have been shown to contain tissues derived from the donor EC cell. These workers have further been able to demonstrate that the EC cell's derivatives in the normal mouse retain their enzyme-deficient phenotype. None of the chimaeric mice has Lesch-Nyhan symptoms however, despite extensive colonisation of the nervous system by donor tissue in some cases. One further point of interest is that teratocarcinoma-derived blood tissue is rare in these chimaeras, when compared with chimaeras containing tissues from the wild-type version of the same cell line. This could possibly be due to the fact that HPRT⁻ blood cells are at a selective

disadvantage in the normal mouse circulation. This compares with human HPRT⁻/HPRT⁺ female heterozygotes where, due to X-chromosome inactivation, blood cells express either the HPRT⁻ or the HPRT⁺ phenotype, and this has been shown to result in a selective advantage for the HPRT⁺ cells, resulting in an overall HPRT⁺ blood phenotype (Nyhan *et al. Proc. natn. Acad. Sci. U.S.A.* **65**, 214; 1970).

Although the results so far have seemed very promising, a number of important problems have emerged. If a case is to be made for the use of teratocarcinomas as a way of studying normal development, either on their own or in chimaeric combinations with normal mouse embryos, it must be established that the behaviour of an EC cell in a blastocyst parallels that of its normal embryonic equivalent. On the basis of the available evidence, EC cells cannot be said to be the equivalent of the inner cell mass (ICM) cells of the normal embryo. If ICM cells are injected into a mouse blastocyst the resulting chimaeric adult has contributions from the injected cell in nearly all the tissues that have been examined (Ford, Evans & Gardner *J. Embryol. exp. Morph.* **33**, 447; 1975). In addition the chimaeras obtained from ICM injections show a very fine-grained intermingling of donor and host tissue (this is particularly noticeable in the coat, for example). In general, chimaeras from injected EC cells have EC contribution in few tissues, and the EC cells tend to occur in much larger patches compared with ICM cell chimaeras.

Another problem, especially in view of the potential practical applications of these animals in the study of human disease, is that there is only one published report of EC cells differentiating into functional gametes (Mintz & Illmensee *Proc. natn. Acad. Sci. U.S.A.* **72**, 3585; 1975). In this case the EC cells were taken from an *in vivo* ascites teratocarcinoma. No germ-line chimaeras have been reported from blastocysts injected with EC cells from cultured teratocarcinoma cell lines. This is not encouraging, especially when this one animal is set against the total number of blastocysts injected with EC cells. It is not yet clear why most EC cells fail to form functional gametes. One possibility is that most of the teratocarcinoma lines which have been tested are karyotypically abnormal. It is known that most gross karyotypic changes in the normal mouse result in sterility, and it is conceivable that the germ line has some form of 'self defence' mechanism to prevent the inheritance of deleterious chromosomal abnormalities. This problem could be studied with the use of a number of karyotypically normal teratocarcinoma cell lines which have been established.

Another difficulty is that there is no way of controlling the sort of tissues that an

EC cell will form in a normal embryo. This means that comparisons between human disorders and teratocarcinoma chimaeras are very difficult, unless one tissue alone, by chance, happens to be derived in part from the donor EC cell.

The use of teratocarcinoma cell chimaeras as a practical tool in developmental biology and medicine has enormous potential. The results in this area are exciting, but a lot more information is needed on the nature and behaviour of EC cells in the normal mouse embryo before the 'man-made mouse' takes its place as a standard laboratory research tool. □

Beads and skeletons

from Graham Cowling

THE dramatic structural changes that occur in the nucleus of a cell as it prepares to divide, have fascinated biologists for over a century and yet, in terms of molecular interactions, the structure of the eukaryotic chromosome is still far from understood. The revealing discovery of beaded chromatin structure has led to rapid progress in the study of DNA-histone interactions. These advances have, in turn, resulted in various models being proposed to explain how arrays of nucleosomes, the basic building blocks made up to DNA and histones, can be supercoiled to form the higher order structures seen *in vivo* (see for example, Finch & Klug *Proc. natn. Acad. Sci. U.S.A.* **73**, 1897; 1976). Are these aggregates of nucleosomes sufficient to give a complete understanding of the overall organisation of chromatin in chromosomes? It seems unlikely from the recent results of U. K. Laemmli's group which suggest that a network of non-histone proteins is responsible for organising chromatin in metaphase, and possibly interphase, chromosomes.

Laemmli and coworkers (*Cell* **12**, 805; 1977) showed that, after histones and the majority of non-histone proteins had been removed from HeLa metaphase chromosomes by treatment with dextran sulphate and heparin, the chromosomal DNA remains in an organised and reasonably compact structure. These histone-depleted structures sedimented in sucrose gradients as a broad peak (4,000–7,000 S) at a density range distinct from free DNA or intact chromosomes. The isolated complexes, stained with ethidium bromide, appeared under the fluorescent

Graham Cowling is in the Department of Biophysics, King's College, London.

microscope as a central core with the morphology of intact chromosomes, which was surrounded by a halo of DNA. Each chromatid was still paired with its sister chromatid although the central structure was 2–3 times the size of the original chromosome. These histone-depleted structures could be dissociated in 4 M urea, but remained intact when isolated in gradients containing 2 M sodium chloride. Mild treatment with trypsin or chymotrypsin also destroyed their morphology.

Gel electrophoresis of the proteins in the histone-depleted chromosomes revealed the presence of about 30 non-histones, most of which had molecular weights greater than 50,000. No bands with the mobilities of either histones or actin, previously found in nuclei (Douvas *et al. Proc. natn. Acad. Sci. U.S.A.* **72**, 3902; 1975), were seen but one of the three major bands had a molecular weight close to that of the related fibrous protein β -tubulin, but as yet not identified as such.

Electron micrographs of these histone-depleted metaphase chromosomes (Paulson & Laemmli *Cell* **12**, 817; 1977) show in more detail how the core of condensed material retained the original chromosomal morphology and was surrounded by many loops of DNA. In some pictures individual DNA loops, ranging in size from 30–90 kilobases, could be seen leaving the core and returning to a position adjacent to their exit points. No supercoiling of the DNA loops was seen, but as Paulson and Laemmli point out, the preparation of such complexes probably allows nicking of the DNA strands to take place. When histones were removed from the chromosome spreads by prolonged washing with 2 M sodium chloride, reported to remove all histones, similar structures to those prepared by polyanion methods were seen by electron microscopy. It therefore seems unlikely that traces of histones were responsible for retaining the morphology seen.

Concurrent work by Laemmli's group (*Proc. natn. Acad. Sci. U.S.A.* **74**, 4937; 1977) has shown that by treating intact HeLa metaphase chromosomes with micrococcal nuclease, a protein 'scaffold' independent of DNA can be isolated on sucrose gradients. The proteins in these scaffolds were identical to those found in the histone-depleted chromosomes. Even more surprising was that the scaffold's shape resembled that of the original metaphase chromosomes, with pairs of sister chromatids still attached by the edges of the scaffold. This suggests that a network of non-histone proteins rather than DNA governs the overall packaging of the chromosome. Another intriguing aspect of these nuclease-prepared scaffolds is that they contain

small pieces of tightly bound DNA. If scaffold proteins recognise a few specific sequences of DNA, considering the number of loops seen in these structures, any such sequences must be at least called repetitive. Perhaps this represents an exciting new system for the study of DNA-protein recognition mechanisms.

Previous models for chromosome structure have been suggested by electron micrographs of intact chromosomes and recently complemented by the study of the nucleosomal packaging of chromatin. In most part non-histones have been left out of models that use a central core of chromatin to organise the rest of the DNA fibres into loops around its axis (see Stubble-

field & Wray *Chromosoma* **32**, 262; 1971). Laemmli's group has proposed a new model which uses a network of non-histone proteins to both trace out the shape of the chromosome and to organise the surrounding DNA into loops. In histone-depleted chromosomes the masses of free DNA strands are probably the result of unravelling the solenoid or 'superhelix' arrays of nucleosomes that surround the scaffold. If this proves to be a general model for metaphase and, possibly, interphase chromosomes further investigation of the scaffold proteins and their changes during the cell cycle may allow a greater understanding of how the cell organises replicated DNA before mitosis. □

Electrical conduction in discontinuous metal films

from Robert M. Hill

DISCONTINUOUS metal films deposited on insulating substrates have been of interest to solid state physicists for many years.

Nucleation theory supports the experimental observation that the initial condensate of a vapour on a clean surface is in the form of small particles and not as a uniform, constant thickness layer. This applies not only to water vapour on a cool substrate but to the deposition of metallic vapours under vacuum conditions. It is only when the average thickness of the deposit is more than some critical thickness that physical continuity is established within the metallic film. The structure of the deposit, in the initial stages of growth, is sensitive to many of the physical parameters of the system. A metal-substrate combination that gives a low surface mobility for the condensate will yield a film formed from small particles closely packed on the surface. Typical dimensions can be as small as 5 nm particle sizes and less than half this figure for the interparticle spacings. These dimensions can be varied by changing the physical conditions during deposition, a higher substrate temperature giving larger particles and wider spacings.

In the classification of solids as metals, insulators and semiconductors the quasi-two dimensional particulate film has been found, experimentally, to bridge the region between insulating behaviour, when the interparticle spacings are large, and metallic behaviour, when the particles have grown sufficiently to aggregate

and form metallic, percolative, paths. Between these limits a semiconductor-like behaviour is seen but the activation energy of the current is generally very small, and usually less than 0.1 eV.

The transition between the semiconducting and metallic regions can be observed by a change in the sign of the temperature coefficient of resistance. The low temperature coefficient region is of obvious interest as an electronic circuit resistor. Three-dimensional particulate films can be prepared by the co-deposition from vapours of metallic and insulating components, the cermet film, and show properties similar to the two-dimensional structures, with an improved stability. The cermet structure has been used as resistors for almost 20 years.

As with most surface phenomena the properties of a discontinuous metal film are strongly dependent on the ambient conditions. Indeed the films are at best only metastable and can be annealed at temperatures in excess of the deposition temperature with a consequent change in the physical structure and hence in the structure-sensitive properties. This makes physical examination, and the correlation of the structures with other properties, difficult. In a recent review of the field Morris and Coutts (*Thin Solid Films*, **47**, 3; 1977) have critically examined the published information and reach the conclusion that the nature of charge transport in particulate films is, as yet, poorly understood. Much of the information is contradictory and in very few cases has sufficient detail been given to

Robert M. Hill is Reader in Physics at Chelsea College, London.

allow evaluation and comparison of the physical phenomena involved.

Initially the activated behaviour was taken to be evidence of thermal ionisation effects but the magnitudes of the energies involved were found to be roughly inversely dependent on the particle sizes. It is now accepted that coulombic interactions do form the basis of this energy but that the physical size of the particles cannot be neglected. The transport of charge from one particle to another can occur either through the insulating substrate or through the free space. Examination of the energies involved shows that the former is more probable, and the strong dependence of the resistance of the films on the particle spacings indicates that the mechanism is likely to be due to quantum mechanical tunnelling. Supporting evidence is found from the detailed temperature dependence of the low field conductance and from the electric field dependence of the current at high fields. However the magnitudes of the observed currents do not correlate with the observed structure. The obvious explanation of this discrepancy is that the structure is not uniform, and may not even be homogeneous. Electron microscopical investigations show that this is indeed the case, with wide distributions both of particle size and spacing. Only recently have attempts been made to introduce the distributed nature of the parameters into the theoretical models of transport (Abeles *et al. Adv. Phys.* **24**, 407; 1975; Hill & Coutts *Thin Solid Films* **42**, 201; 1977; Leaver *J. Phys. C* **10**, 249; 1977). The problem is undoubtedly difficult for it is necessary to use a percolative approach in which easy transport paths through the material are given a probability weighting dependent on their conductance. But even within these paths the individual particles and gaps are distributed. Recent work has shown that at high temperatures a clear activated region should be observed when transport is between nearest neighbour particles, but at low temperatures where the number of easy paths decreases the activation energy will become temperature dependent as carriers have to be transported to more distant neighbours. This second region of behaviour is similar to the variable range hopping region in amorphous semiconductors (Mott *Festkörperprobleme* **9**, 22; 1969) or in impurity band conduction in heavily doped and compensated semiconductors (Mott & Davis *Electronic Processes in Non-Crystalline Materials*; Clarendon Press, Oxford, 1971) except that the size of the active centres is much larger and hopping can only occur between the supply particle and a limited number of nearer neighbours. Experimental verification of the temperature dependence of the activation energy is not available yet as very few films have been examined at sufficiently low temperatures to observe the full transition in behaviour

(Abeles *et al. op. cit.*).

A range of interesting physical effects has been observed in discontinuous and quasi-continuous metal film structures (Morris & Coutts *op. cit.*). A number of these could form the basis of practical sensors but as electrical charge transport

is central to the understanding of these effects it is essential that our understanding of the process, or processes, involved should be placed on a firm foundation. In their review Morris and Coutts clearly show that this is not the case. □

New light on Phobos, the inner satellite of Mars

from G. Fielder

PHOBOS is a dynamic body with a difference: it is one of the smallest natural satellites in the Solar System, and completes one revolution of Mars every 7.65 hours at a mere 9,000 km from the planet's centre. Recent discoveries about Phobos have opened up exciting prospects for students of planetary science. First, close up views of intact small bodies in the Solar System are few; and, second, exploration of numerous planetary bodies (including natural satellites and asteroids) which offer, collectively, a range of size and a large spread of solar distance is of the highest importance in unravelling the early history and origin of the planetary system.

Two quantities—the radius and mass—are fundamental in planetology. Until the Mariner spacecraft photographed Phobos, in 1969 and (better) in 1971, its

mean radius had to be estimated by assuming an albedo and measuring the amount of sunlight that the satellite scattered back to Earth. And until the Viking Orbiter I was Doppler-tracked, in 1977, to measure its accelerations relative to Phobos, the mass of Phobos could not be computed. Once the mass and radius of a planet are known, the mean density can be calculated: that is important because it is related to the planet's composition. Phobian mass determinations, by two groups of researchers (Tolson *et al. Geophys. Res. Lett.* **4**, 551; 1977; Christensen *et al. Geophys. Res. Lett.* **4**, 555; 1977) who used different methods of processing the same raw data, are therefore of very great interest. The mean value is close to 1.0×10^{16} kg.

But Phobos is not a sphere. There is no reason why it should be since, on such a small body, the particles experience little in the way of mutual gravitational attraction. Photographs (Veverka *et al.*

G. Fielder is at the Lunar and Planetary Unit, University of Lancaster.

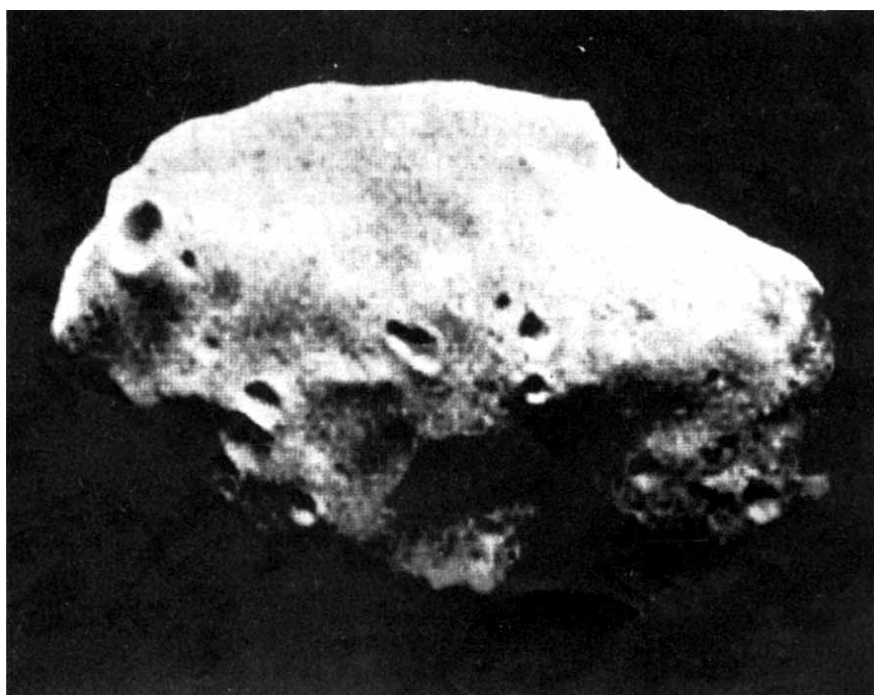


Fig. 1 NASA Mariner 9 photo of Phobos showing craters.

Icarus 23, 206; 1974) taken by Mariner 9 showed Phobos (Fig. 1) to be intensely cratered and highly irregular in shape. A few of the craters, such as the 10-km diameter Hall, have widths approaching the mean radius of Phobos itself, and these craters contribute markedly to the overall irregularity. Even a triaxial ellipsoid is not too good a fit to the surface of Phobos, which seems to have semi-axes of about 13.5 (pointing towards Mars), 10.7 and 9.6 km, respectively. The volume of Phobos, corrected for the volume of the craterial depressions (mainly, the largest ones) below the ellipsoid, is about $5 \times 10^{12} \text{ m}^3$; and thus the density turns out to be only $2,000 \text{ kg m}^{-3}$ (2 g cc^{-1}). It is a surprise because it is so small: some scientists view Phobos as a captured asteroid, and the mean density of asteroids is likely to be more nearly $3,500 \text{ kg m}^{-3}$.

What, then, can be deduced about the composition of Phobos? The albedo (Short *Planet. Geol.*, Prentice-Hall, 1975) of Phobos is similar to that of chondritic material, but this has a density that is higher than $2,000 \text{ kg m}^{-3}$. However, the observed low mean density of Phobos can be synthesised using a suitable mixture of carbonaceous chondritic material (which itself contains 20% by weight of water) and voids, or low density materials such as ice. Under the prevailing conditions (the surface temperature of Phobos may be calculated, using an albedo of 0.05, and attains some 230 K or -40°C) ice could exist inside the satellite provided that the interior was not warmer than predicted on the basis of solar heating alone. However, present-day heating of Phobos is not unlikely. Phobos actually resides within the Roche limit for a fluid body which, had it replaced Phobos, would have disintegrated. Phobos remains in one piece because it has more mechanical strength than a fluid. Yet, if the tiny moon were to move only 3,700 km closer to Mars, then even Phobos would probably be torn apart. With the present Mars-Phobos configuration, the materials of Phobos are being subject to stresses of the order of 10^4 N m^{-2} . This kind of stress level is insufficient to fracture solid rocks as we know them on Earth, but new Viking Orbiter photographs (Fig. 2) of Phobos show that its underdense materials have indeed been tidally fractured: the surface displays striking arrays of lineaments (actually approximating to arcs of ellipses), and these lineaments trend in just those directions predicted by the theory of tidal fracturing of a natural satellite which is gravitationally bound to face its primary (Gash, thesis, University of Lancaster, 1973). Even though the orbital eccentricity (0.02) of Phobos leads to rather small changes in the tide-raising force, the components of this force also change in magnitude as a result of the physical libration of Phobos (Duxbury *Icarus* 23, 290; 1974). An

Photo: NASA

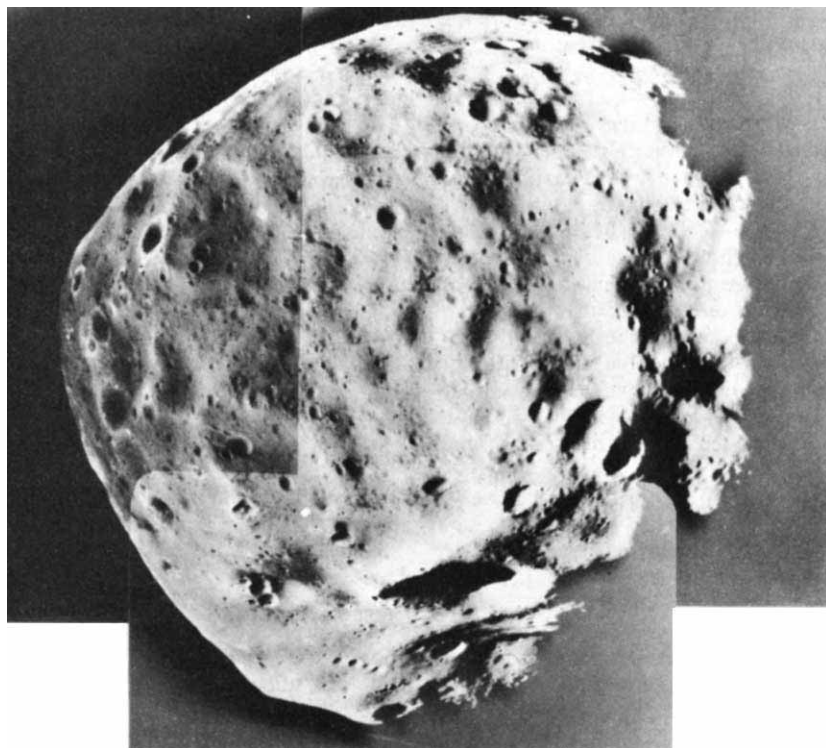


Fig. 2 NASA Viking Orbiter photo of Phobos showing crater chains. A series of craters runs horizontally in the picture, which is parallel to the orbit plane of Phobos.

effect of such tidal stressing is to dissipate gravitational energy as heat—in this case, mainly along the fracture lanes. The underdense rock conductivity will be small and it is conceivable that repeated stress changes will lead to the melting of the materials along the fractures. The volatiles will have left through the impact-pulverised upper layers of Phobos and the loosely bonded surface materials will have subsided into the vacant spaces to produce crateriform depressions along the

lengths of the fractures, as observed (Fig. 2). Since these lineaments, or crater chains, seem to dissect all the larger craters on Phobos, it is clear that the fractures have been worked more recently than the most important phase of impact cratering.

Further studies of the new data are likely to throw some light on the orbital evolution of Phobos. Whether or not Phobos is a captured asteroid may then become clearer. □



A hundred years ago

At the meeting of the Linnean Society on Thursday last it was unanimously resolved to send a congratulatory letter to von Siebold on the occasion of his jubilee. This graceful act, however, brings into prominence the neglect of the Society to take any notice of the Linnean centenary, the celebration of which in Sweden, Holland and Germany were recently noticed in our columns. Of course the excuse may be urged with some force that such formalities are foreign to English habits, but perhaps an exception might have been allowed in the case of a Society which bears the name and jealously guards the collections, books and manuscripts of the great naturalist. Perhaps however, another reason may be found in the fact that the constitution of the Society places the initiative in every case in the hands of

officers whose tenure of office is practically indefinite, and who are not very accessible to any impulses of enthusiasm from the general body of the Society even if there were any permissible way by which expression could be given to them. Some disquieting rumours as to the present condition of the Society's business affairs, coupled with its rather troubled history during the past few years, seem to point to the desirability of some changes in its mode of government which would bring the executive into closer relation with the general body of Fellows.

AMONGST the exports of Corsica it is said that there are annually between 350,000 and 400,000 blackbirds (*merles*) which are sent to this continent. They visit Corsica in vast numbers each winter to feed on the berries of the myrtle and arbutus, with which the mountains are covered. In the month of December they become very fat and the flavour and the perfume given to their flesh by their food causes them to be much esteemed by the *gourmets* of Paris. A *pâté de foie de merle* is a great delicacy.

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articles

Seafloor spreading in the western Gulf of Aden

R. W. Girdler & P. Styles

School of Physics, The University, Newcastle upon Tyne, UK

Interpretation of a new magnetic survey suggests that the evolution of the Gulf of Aden involved at least two phases of seafloor spreading. A recent phase from about 5 Myr ago to the present is well established and an early phase from about 30–15 Myr ago seems most likely.

A DETAILED geophysical survey was carried out in the western Gulf of Aden in 1975 aboard RRS Shackleton, mainly to discover more about the seafloor spreading history of this region¹⁻⁷. Satellite navigation was used throughout. Here we report the main results of the magnetic survey and conclude that there were at least two spreading phases. We also give a tentative location for the Arabia–Danakil–Somalia triple junction.

The survey

Sixteen profiles were obtained (Fig. 1) about 10 km apart along the direction N32°E. This direction is presumed to be the most likely spreading direction between the Somalia and Arabia plates and is computed from the best fit of the shorelines. All the seismic refraction profiles⁸ show oceanic structures. These are shown in Fig. 1 and will be used in the interpretation.

Two profiles (Fig. 2) illustrate the nature of the anomalies and their relationship to bathymetry. Note that (1) the anomalies over the axial rift and ridge contrast with those over the rest of the Gulf. The former reach more than 2,000 nT, whereas the latter typically reach only 400 nT. (2) Profile *F* shows anomalies symmetrically disposed about the axial rift valley. (3) Profile *B* shows more anomalies to the south of the axial rift than to the north.

Interpretation

Laughton *et al.*⁷ tentatively identified anomaly 5 and suggested continuous spreading over the past 10 Myr. In contrast, it has been difficult to identify anomalies over the Red Sea and establish continuous spreading beyond 3 to 4 Myr ago^{4,5,10,11}. We suggested^{12,13} at least two stages of Red Sea spreading, the first in the Oligocene, the second in the Plio–Pleistocene. As the Gulf of Aden and Red Sea are considered to result from the anticlockwise rotation of Arabia with respect to Nubia and Somalia^{3-5,14-16} and there is no evidence to a first order for shear between Nubia and Somalia, the spreading histories of the Gulf of Aden and Red Sea should be similar. The two interpretations of the magnetic anomalies are, therefore, in conflict.

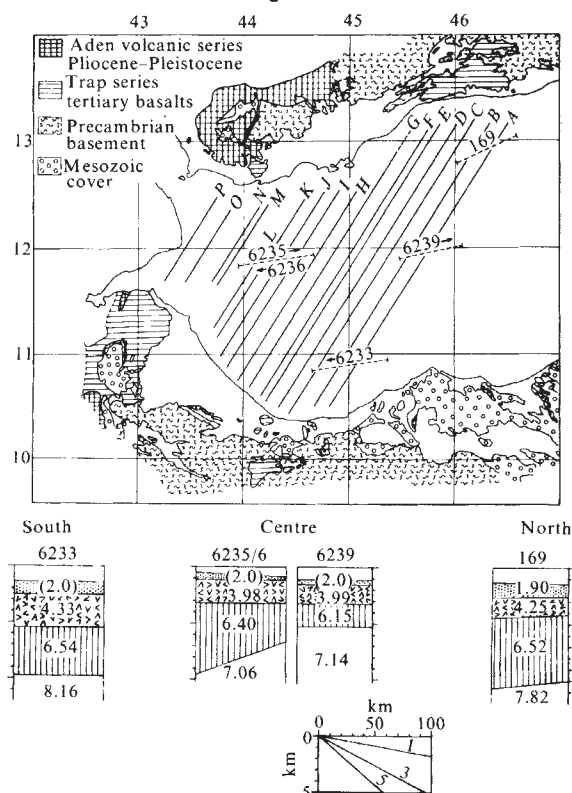
The continuous spreading model of Laughton *et al.*⁷ for the western Gulf of Aden is shown in Fig. 3. It is difficult to correlate the anomalies beyond 3–4 Myr both in number of anomalies and in their amplitudes. We therefore consider an alternative interpretation involving discontinuous rather than continuous spreading.

The correlation of the anomalies back into the Pliocene is good. At the end of the Miocene there was a spectacular change in the conditions of sedimentation in the Red Sea, probably because of some major tectonic event. Possibly, therefore, the most recent phase of spreading started at the time of the

Miocene–Pliocene boundary (about 4.9 Myr ago). Before this there seems to have been a relatively quiet period during which the main trough of the Gulf of Aden accumulated up to 1.5 km of sediment according to the seismic refraction data. For the earlier history, we may look at the geology of neighbouring Somalia and Arabia⁶. The stratigraphy of the northern and southern sides can be closely correlated up to the end of the middle Eocene. After this, sediments are confined to the littoral zones. The main trough could thus have formed between the beginning of the Upper Eocene and the end of the Miocene. The magnetic profiles were therefore compared with synthetic profiles generated for the period from 40–5 Myr.

Figure 4 shows the interpretation for the symmetrical profile *F*. A remarkably good fit can be obtained when the period 30–17 Myr is chosen for the early spreading phase. For the recent phase of spreading, a dipping layer 2 model is used in accordance with the cooling law for new oceanic crust¹⁸. For the older phase the dip is considered negligible, but a much better agreement is obtained if the magnetisation is assumed to reside in layer 3. If layer 2 is included there is too much high

Fig. 1 The locations of the sixteen total intensity magnetic profiles (A–P) in the western Gulf of Aden. The dashed lines with numbers are seismic refraction profiles⁸, the sections for which are also shown (velocities in km s⁻¹). Neighbouring geology is given.



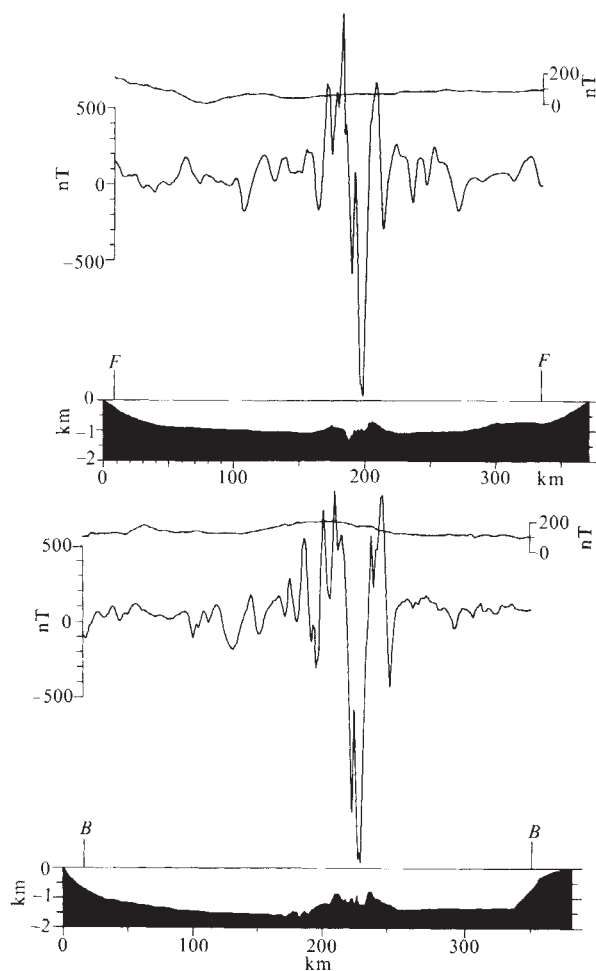
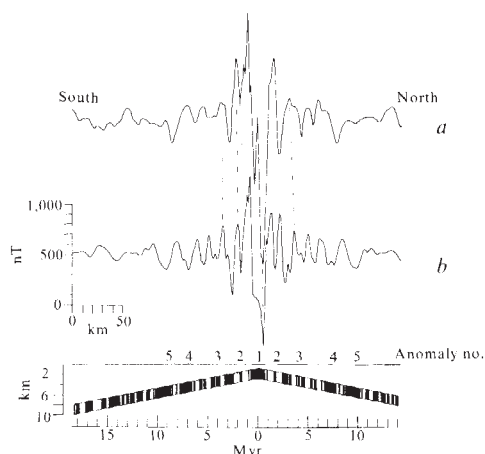


Fig. 2 Two examples of the total intensity magnetic profiles. The regional field removed was the International Geomagnetic Reference Field 1975 (ref. 9) for 1975.25 (the date of the survey) and corrections (upper profiles) for time variations of the Earth's field were applied using base station records from Djibouti supplemented by magnetograms for H from Addis Ababa. Note the change in character of the anomalies on both sides of the axial rough zone. Vertical exaggeration 23:1.

Fig. 3 The continuous spreading model of Laughton *et al.*⁷ for the western Gulf of Aden together with (a) an observed profile (F) for comparison with (b), the synthetic anomalies, spreading rate 1 cm yr^{-1} generated using the reversal timescale of ref. 17.



frequency signal on the synthetic profile when the depth constraints of the seismic refraction lines are used. This suggests that the magnetisation of layer 2 decays with age and layer 3 takes over in importance. This has also been noticed by Roeser¹⁹. Alternatively, the model may be too simple; for example, Blakeley²⁰ has proposed an age dependent two layer model for marine magnetic anomalies. This will be discussed further elsewhere.

For profile B (Fig. 5) there are more anomalies to the south of the axial ridge than to the north. When the contrasting amplitudes of the anomalies for the new and old phases of spreading are considered, it seems that the new phase did not start in exactly the same place as the old one stopped. The anomalies can be interpreted in harmony with the interpretation for profile F, provided the former spreading axis is some distance south of the new spreading axis. It seems that the first phase of spreading stopped about 15 Myr ago, that is at the end of the Lower Miocene.

Consideration of all the profiles shows that there are two transform faults between profiles B and F. Interpretation of B and F suggest that spreading to the west of the transform between profiles D and E stopped and started in the same place but spreading to the east of this transform restarted some distance to the north of where it stopped (Fig. 6).

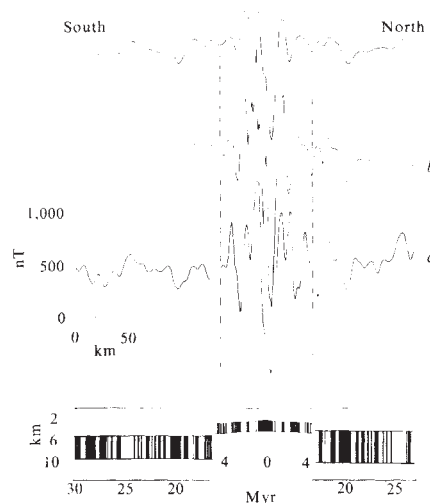


Fig. 4 Seafloor spreading model to explain the magnetic anomalies for profile F. (b) Shows the observed anomalies and (a) the upward continued anomalies (1.83 km). The latter is helpful in identifying various marker features. (c) Shows synthetic anomalies considered to give the best fit. Spreading rates: centre 1 cm yr^{-1} ; S limb 1 cm yr^{-1} ; N limb 1.13 cm yr^{-1} . These have been computed using the usual simplifying assumption of vertically sided blocks. The seismic refraction profiles (Fig. 1) have been used for the depths and thicknesses of the magnetised layers.

Discussion

The evidence suggests the following history of the Gulf of Aden. First, major movements probably occurred 40 Myr ago; by the end of the Upper Eocene the Gulf of Aden downwarp was in existence and the first major phase of seafloor spreading began 30 Myr ago (beginning of Upper Oligocene). This phase continued until about 15 Myr ago (end of Lower Miocene). There was then a quiet period during which up to 1.5 km of sediment accumulated. At the end of the Miocene, spreading recommenced continuing to the present. We emphasise that this interpretation is the simplest which fits the observations; the possibility of more than two spreading phases cannot be excluded.

The dates for the early stage of seafloor spreading (30–15 Myr) agree well with the dates of basaltic activity in neighbouring regions²¹. They differ from the dates (41–34 Myr) deduced for the Red Sea¹². At that time, the choice of model was constrained by the dates (36.4 ± 2 , 36.7 ± 2 , 30 Myr) for rocks in

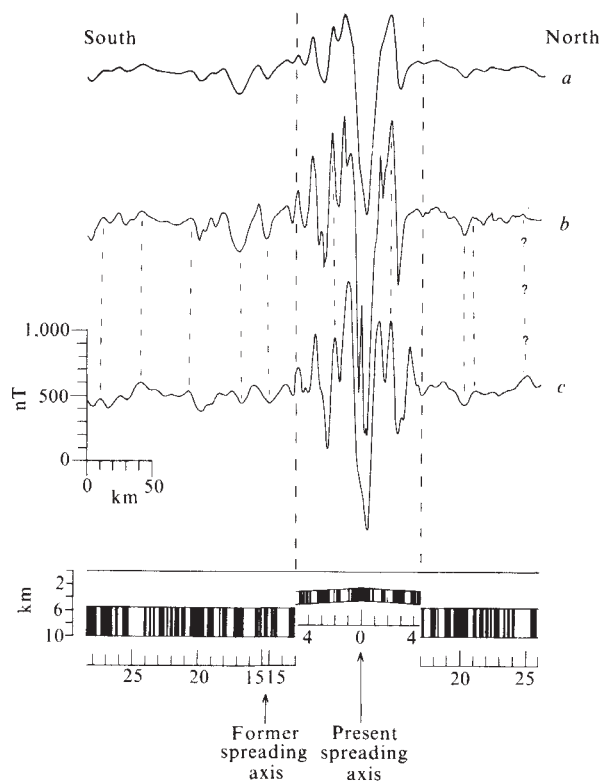


Fig. 5 Interpretation of profile *B*. Note the smaller amplitude of the observed anomalies on the northern side due to the proximity of a transform fault. (a) Shows the anomalies upward continued to 1.83 km, (b), observed anomalies at sealevel and (c), synthetic anomalies with spreading rate 1 cm yr⁻¹.

Red Sea boreholes²². These are now known to be in error (Nelson, personal communication) and a re-examination of the Red Sea magnetic data (to be published) shows that the magnetic anomalies for the Red Sea can be interpreted in harmony with the seafloor spreading history of the western Gulf of Aden.

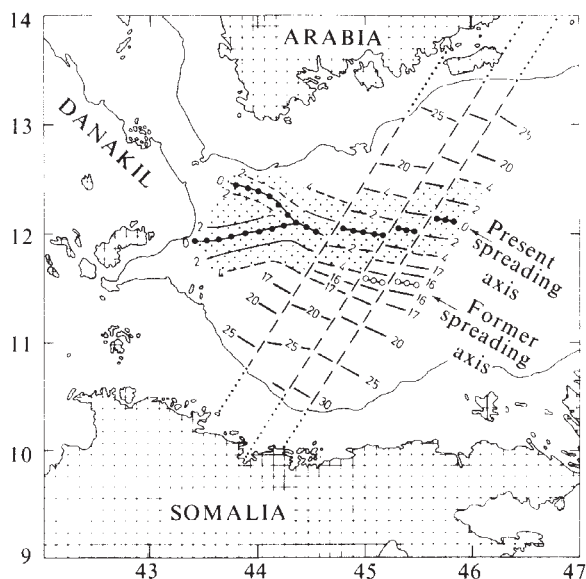


Fig. 6 Isochrons (Myr) deduced from magnetic anomalies for the western Gulf of Aden. The present spreading axis (zero isochron) is shown by a chain of solid circles and the former spreading axis (~15 Myr isochron) is shown by a chain of open circles. The three transforms are shown by dashed lines and their possible extensions by dotted lines. Land over 3,000 feet (914 m) is cross-hatched.

This has several features in common with an early interpretation by Laughton² who suggested that the development of the Gulf of Aden was a consequence of two distinct phases of continental drift. In phase 1, the continental crust was envisaged to be stretched, thinned and intruded by basaltic dykes. After this there was a period of 'reduced movement' during which the main trough accumulated 0.5 to 1.5 km of sediment. This was followed by phase 2 in which new oceanic crust formed the central rough zone. The new interpretation replaces Laughton's phase 1 by a major period of seafloor spreading over a period of some 15 Myr through the Upper Oligocene and Lower Miocene.

Plate geometry

Figure 6 summarises the magnetic interpretations. From most of the magnetic profiles, it is possible to identify the positions of the 2 and 4 Myr isochrons and these are shown. The region between the 0 and 4 Myr isochrons is stippled, illustrating the minimum extent of the new phase of spreading. The strike of the spreading axis is N110°E. Previously⁷, the transforms and offsets had not been recognised and the spreading axis was considered to be continuous with a strike of N 80°E. The new interpretation is consistent with the ridge-transform pattern further east⁷ and with the direction of separation determined from the fit of the coastlines^{15,16}. In the west, the present spreading axis bifurcates, one branch going towards the Straits of Bab-el-Mandeb and the other towards the Gulf of Tadjura. The bifurcation marks the present location of the triple junction for the Somalia-Arabia-Danakil plates.

For the older spreading phase, the 16, 17, 20 and 25 Myr isochrons are shown. In the extreme south, it is possible to identify tentatively the 30 Myr isochron (close to the Somali shore). This implies that the minimum separation between the Somalia and Arabia plates is 380 km and that a considerable proportion of the Arabia and Somalia coastal plains to the west of 45°E must be underlain by 20–30-Myr-old oceanic crust.

In the east of the survey area, the new spreading axis is about 75 km north of the old spreading axis. The transforms associated with the old and new spreading phases are approximately collinear and the isochrons are all approximately parallel. This implies that within the limits of accuracy of navigation, the poles of rotation for the two phases of movement must be very similar and close to the pole of rotation for the total movement, that is 26.5°N, 21.5°E^{14,16,23}.

We thank Captain P. Warner and the officers and crew of the RRS Shackleton, also Dr J. C. Lepine (Djibouti) and P. Gouin (Addis Ababa) for help with base station magnetometer records; and A. S. Laughton and R. B. Whitmarsh (I.O.S. Wormley) for interest and encouragement. The work was financed by Natural Environment Research Council Grant GR/3/2467.

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- Girdler, R. W. & Peter, G. *Geophys. Prosp.* **8**, 474–483 (1960).
- Laughton, A. S. *Phil. Trans. R. Soc. A* **259**, 150–171 (1966).
- Laughton, A. S. *Geol. Surv. Can. Paper* 66–14, 78–97 (1966).
- Girdler, R. W. *Geol. Surv. Can. Paper* 66–14, 65–77 (1966).
- Girdler, R. W. *Hot Brines and Recent Heavy Metal Deposits in the Red Sea* (eds Degens, E. T. & Ross, D. A.) 38–58 (Springer, Berlin, 1969).
- Beydoun, Z. R. *Phil. Trans. R. Soc. A* **267**, 267–292 (1970).
- Laughton, A. S., Whitmarsh, R. B. & Jones, M. T. *Phil. Trans. R. Soc. A* **267**, 227–266 (1970).
- Laughton, A. S. & Tramontini, C. *Tectonophysics* **8**, 359–375 (1969).
- I.A.G.A. Division I Study Group *Geophys. J. R. Astr. Soc.* **44**, 733–734 (1976).
- Vine, F. J. *Science* **154**, 1405–1415 (1966).
- Phillips, J. D. *Phil. Trans. R. Soc. A* **267**, 205–217 (1970).
- Girdler, R. W. & Styles, P. *Nature* **247**, 7–11 (1974).
- Girdler, R. W. & Styles, P. *Inter. Union Commis. Geodynam. Rep.* **16** 2, 156–170 (1976).
- Le Pichon, X. J. *geophys. Res.* **73**, 3661–3697 (1968).
- McKenzie, D. P., Davies, D. & Molnar, P. *Nature* **226**, 243–248 (1970).
- Girdler, R. W. & Darracott, B. W. *Comments Earth Sci.* **2**, 131–138 (1972).
- Tarling, D. H. & Mitchell, J. G. *Geology* **4**, 133–136 (1976).
- Sclater, J. G. & Francheteau, J. *Geophys. J. R. Astr. Soc.* **20**, 509–542 (1970).
- Roeser, H. A. J. *Geophys.* **42**, 73–80 (1976).
- Blakely, R. J. *AGU geophys. Mon.* **19**, 227–234 (1976).
- Barberi, F., Ferrara, G., Santacroce, R. & Varet, J. *Inter-Union Commis. geo. Sci. Rep.* **14**, 391–416 (1975).
- Lowell, J. D. & Genik, G. J. *Bull. Am. Ass. petrol. Geol.* **56**, 247–299 (1972).
- Girdler, R. W. & Styles, P. *Earth planet. Sci. Lett.* **33**, 169–172 (1976).

Structural evidence for gene duplication in the evolution of the acid proteases

J. Tang

Laboratory of Protein Studies, Oklahoma Medical Research Foundation, 825 NE 13 Street, Oklahoma City, Oklahoma 73104

M. N. G. James & I. N. Hsu

MRC Group in Protein Structure and Function, Department of Biochemistry, University of Alberta, Edmonton, Canada T6G 2H7

J. A. Jenkins & T. L. Blundell

Laboratory of Molecular Biology, Department of Crystallography, Birkbeck College, University of London, Malet Street, London WC1, UK

X-ray studies of acid proteases indicate a bilobal structure with a well defined active site cleft. An intramolecular two-fold symmetry axis relates two topologically similar domains and the active site residues. A possible mechanism for evolution by gene duplication, divergence and gene fusion is presented.

X-RAY crystallographic studies have shown that proteins are organised into globular domains. Those proteins of molecular weights <25,000 have domains comprising between 100 and 200 amino acid residues. Generally the amino acids in one domain fold individually and are then organised together to give the native tertiary structure¹. Further it is possible that the proteins evolved from smaller functional units by gene fusion; thus lactate, alcohol and glyceraldehyde phosphate dehydrogenases have nucleotide binding domains strongly resembling each other linked to quite different domains which contribute towards substrate specificity^{2,3}. A more primitive nucleotide binding protein may have been a common ancestor³. In other proteins where gene duplication had occurred, it may have been selectively advantageous to follow this by gene fusion leading to a protein with two similar globular domains. We present here evidence for such a mechanism in the evolution of acid proteases leading to a bilobal structure in which an approximate twofold symmetry axis relates two topologically similar domains and the active site aspartate residues.

Acid proteases are so called because they have an optimal catalytic activity at acid pH (for a general review, see ref. 4.) They include mammalian digestive enzymes such as pepsin, chymosin and cathepsin D, microbial proteases such as those produced by *Penicillium janthinellum* (penicillopepsin)^{5,6}, *Rhizopus chinensis* (rhizopuspepsin)⁷, and *Endothia parasitica* (endothiapepsin)^{7,8} and probably certain enzymes involved in mammalian control processes, for example renin, which acts on angiotensinogen.

These acid proteases are characterised by two active site aspartates and most are inhibited by the microbial peptide analogue, pepstatin. They tend to cleave between hydrophobic amino acids but secondary interactions are important in determining specificity⁹. The sequence of porcine pepsin¹⁰ shows that the enzyme has 327 amino acids with the active site aspartates at residues 32 and 215 (see Fig. 1). Pepsin, penicillopepsin⁶ (Fig. 1) and chymosin¹¹ are closely homologous structures and fragments of the sequences of other microbial enzymes¹¹ strongly indicate an homologous series. Medium resolution X-ray analyses of rhizopuspepsin⁷, endothiapepsin^{7,8} and penicillopepsin^{5,6} confirm this structural homology. Preliminary comparisons of the three structures show root mean square deviations between topologically equivalent α carbons^{12,13} (about 200 of each enzyme) of the order of 1.35 Å (data not shown). A stereo drawing of the α -carbon positions of one of these, penicillopepsin, is shown in Fig. 2. The numbering of the

residues is chosen to facilitate comparison with the sequence of pepsin and is given in Fig. 1. The bilobal structure defines an extensive cleft in which the active site residues Asp 32 and Asp 215 are positioned. The two lobes comprise residues —4 to 171, and 177 to 327, connected by a short length of peptide, 172–176. The secondary structure is predominantly β sheet and the general folding is illustrated schematically in Fig. 3. The identity of the residues in the strands is indicated in Fig. 1, and this nomenclature is derived from that described by Hsu *et al.*⁶. It should be noted that Fig. 3 does not indicate the lengths of the β -sheet strands nor of the connecting loops. Furthermore, the extensive sheet is sharply twisted at several points, for example at strands c and m, and this is more clearly illustrated in Fig. 4. The sequences of pepsin and chymosin strongly suggest that they also have similar secondary and tertiary structures.

Internal structural homology

Tang *et al.*¹⁴ noted the remarkable similarity between the residues 30–42 and 213–225 (see Fig. 1) of pepsin. These two stretches of polypeptide include the two active site aspartyl residues at positions 32 and 215. They suggested that this may have been a consequence of gene duplication and fusion, but the pepsin sequence gave no further indications to support this hypothesis. However, consideration of the secondary structure now shows that these residues form similar β structures (c_2 -d and m_2 -n in Fig. 4) and in each case are crossed by a strand (h_1 parallel to c_2 and p_1 parallel to m_2) which gives rise to a ψ (ψ) shaped structure. These parallel strands h_1 and p_1 (see Fig. 1) also have some sequence homology and in each case are about 90 residues towards the C terminus from the active site aspartates. In fact, the similarity is considerably more extensive than this as can be seen from Fig. 3. A twofold symmetry axis positioned between antiparallel strands i and q relates the structure in the N-terminal lobe to that in the C-terminal lobe. In particular the active site aspartates, the parallel pairs of strands in the otherwise antiparallel β sheet, the small sections of helix (h_2 and p_2) and the interstrand connectivities in the lobes are equivalent. This striking symmetry is carried over to a large extent into the tertiary structure, as can be seen from Figs 2 and 4. However, the tryptophan residues thought to be equivalent on the basis of the primary structure¹⁴ are not in symmetry related positions.

In order to quantify this symmetry relationship we have compared the two lobes using a least-squares refinement programme for 'topologically equivalent' residues^{12,13}. This evaluates the symmetry in runs of contiguous sequence using a progression rule. In penicillopepsin 61 residues of each lobe are topologically equivalent, and the root mean square deviation for these is 2.01 Å. The two lobes are shown superposed in Fig. 5. The rotation angle is close to that of a twofold but not exact; it is 174.6° and there is a translation of 1.0 Å along this rotation axis relating the two lobes.

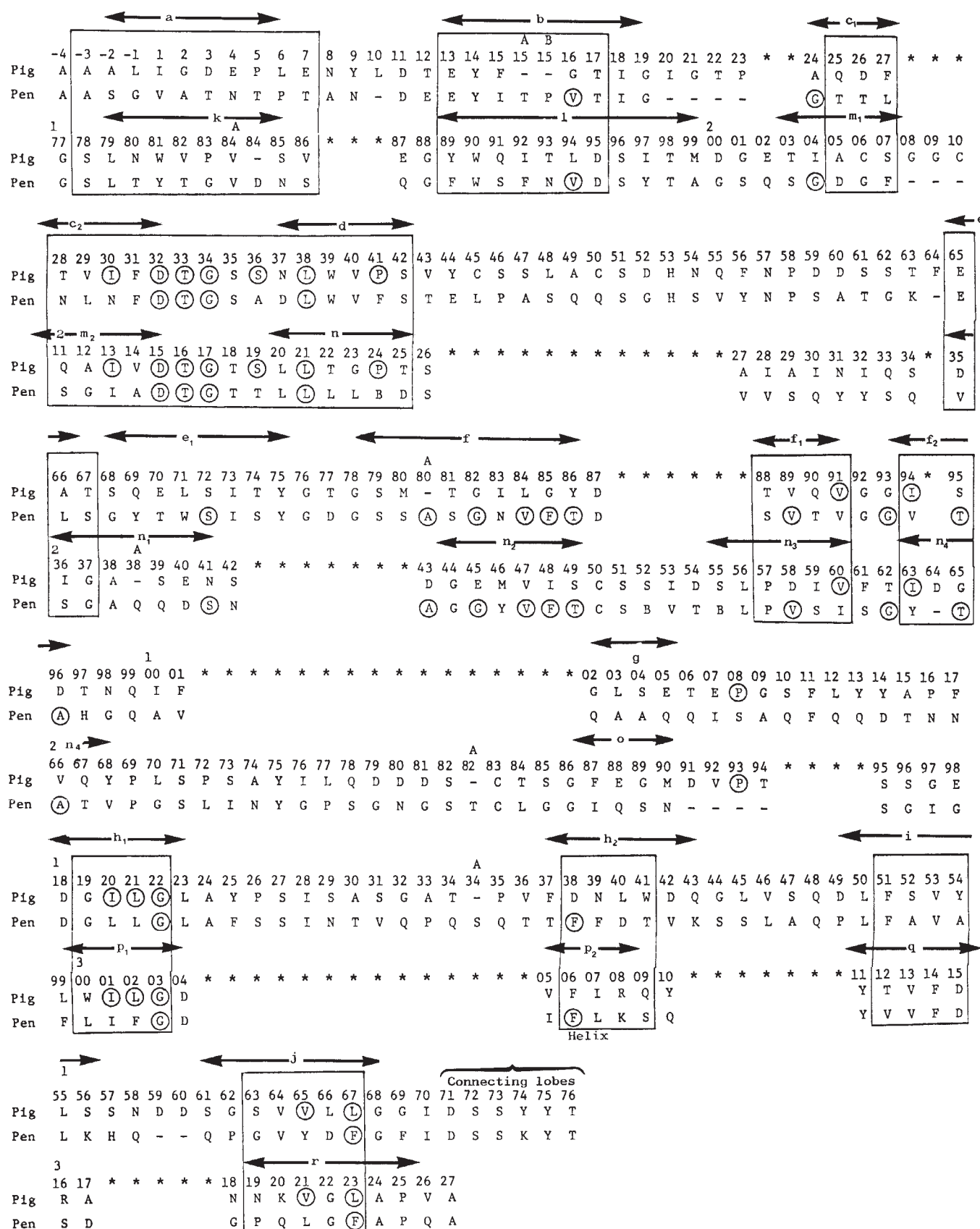


Fig. 1 The sequences of the acid proteases, porcine pepsin and penicillopepsin, aligned to maximise the internal structural homology. Residues in boxes are 'topologically equivalent residues'. Circles indicate residues which are identical in the two lobes. Asterisks identify positions where there is no topologically equivalent residue. The residue numbering is that of porcine pepsin and the strands are lettered to facilitate discussion in the text and identification in the schematic Figs 3 and 4.

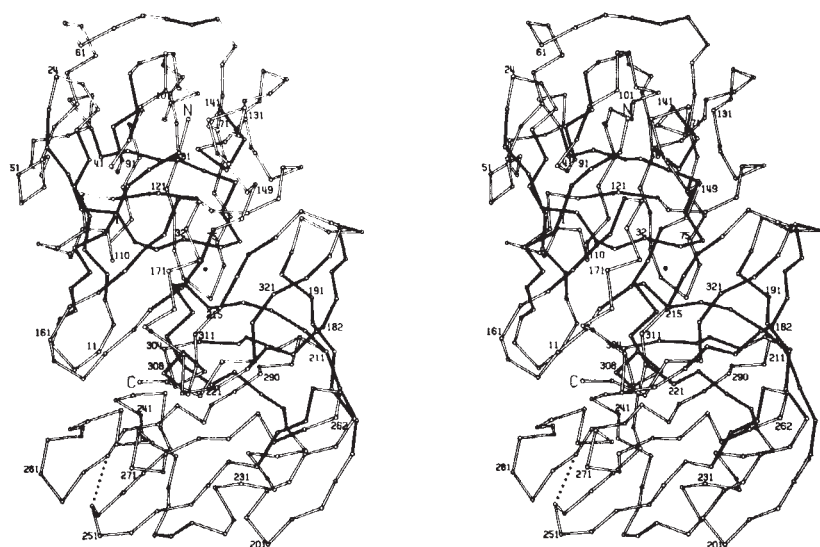


Fig. 2 Stereo drawing of the α -carbon positions of the acid protease, penicillopepsin. The residue numbering given in Fig. 1 is designed to facilitate comparison with the homologous pepsin structure. Structures of the acid proteases, endotheiapepsin and rhizopuspepsin, are very closely related. The enzyme is viewed along the approximate twofold symmetry axis relating the two lobes of the enzyme as described in the text.

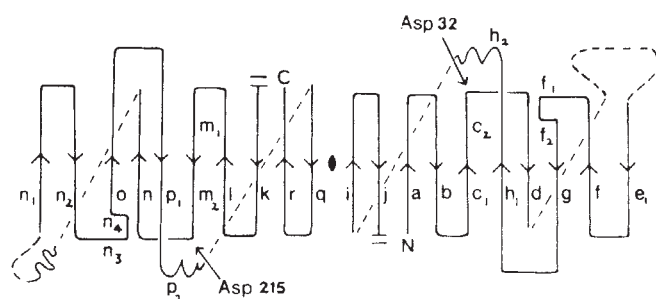
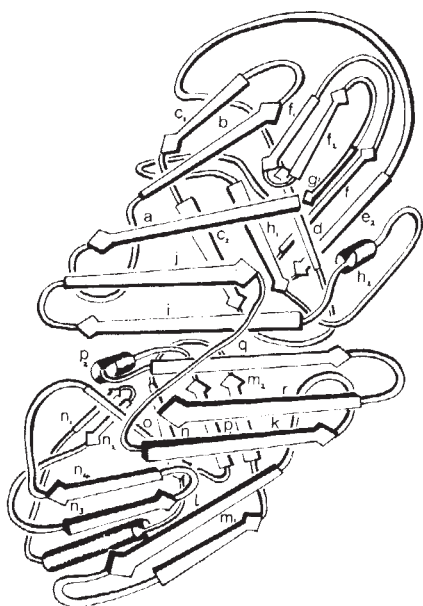


Fig. 3 A schematic drawing of the secondary structure found in the acid proteases. The strand labels are defined in Fig. 1. Strands a-j are in the N-terminal of Fig. 2 and related by an approximate twofold symmetry axis to the strand k-r in the C-terminal lobe. There are rather large angles between some strands such as d and g, n and o; strands c and m bend in the centre as shown in Fig. 4.

Fig. 4 A schematic drawing of the tertiary structure of the acid protease, endotheiapepsin, viewed along the twofold axis. The strand labels are defined in Fig. 1.



The 61 topologically equivalent residues are indicated in Fig. 1 by inclusion in boxes. Assuming porcine pepsin is homologous there are 58 such residues in this enzyme. The sequences are aligned to optimise the topological equivalence. This inevitably leads to gaps which are indicated by asterisks in Fig. 1. These correspond to loops of polypeptide which are present in one lobe but absent in the other.

Several of the differences between the lobes correspond to extensions or deletions of β loops connecting two adjacent antiparallel strands of the β sheet. Thus the loop k-l is a few residues shorter than a-b, and b-c₁ is much less extended than l-m₁, and q-r is less extended than i-j. A more striking example is the loop n₁-n₂ which is seven residues shorter than e₁-f. In other places, for example d-e compared to n-n₁, the chain folds across several strands of sheet and a larger length of less tightly folded polypeptide is inserted on one side (residues 43-55 between d and e). Similar additions and deletions occur in the strands connecting h₁-h₂-i and p₁-p₂-q; in this example rather long stretches of polypeptide are inserted between h₁-h₂ and h₂-i compared to p₁-p₂ and p₂-q, but the two helices h₂ and p₂ nevertheless partially overlap (see Fig. 1).

The insertions which give rise to difference between the lobes are on the surface or available to the surface of the enzyme and do not interfere with the general tertiary fold. Indeed, deletions or additions occur often between penicillopepsin and pepsin in positions which are topologically inequivalent between the lobes; for example the deletion of residues 20-23 (between b and c₁) of pepsin in penicillopepsin. Further, the loop e₁-f which differs from n₁-n₂, is completely on the surface, and these residues in penicillopepsin differ from the equivalent loop in endotheiapepsin and rhizopuspepsin.

Evolution by gene duplication

The two lobes of the acid proteases thus have approximately 35% of the residues in topologically equivalent positions. These results are similar to those observed for the bacterial and pancreatic serine proteases with an important difference. It was first pointed out by Shotton and Watson¹⁵ that in elastase the two domains have a similar fold, but the twofold relationship is such that the polypeptide chain of the C-terminal domain has the opposite polarity to that in the N-terminal domain. This has subsequently been observed in the bacterial serine proteases where the twofold relationship between domains is more exact^{16,17}. It can be concluded, therefore, that the similarity of conformation in the two domains of the serine proteases is a

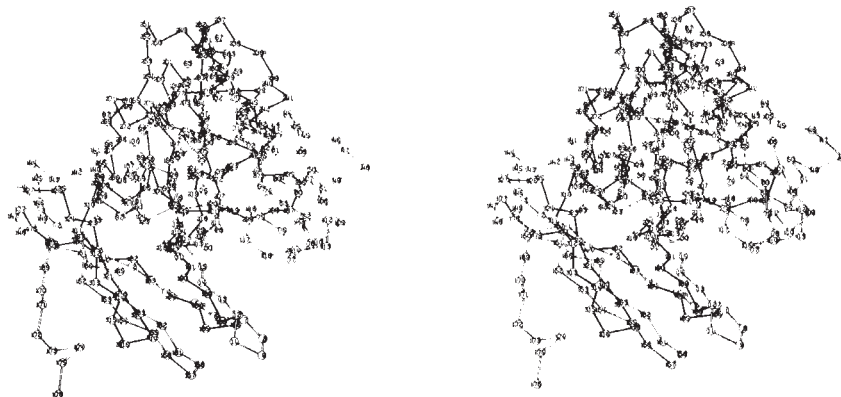
result of specific structural requirements rather than a gene duplication event. This may not be the case for the acid proteases for the twofold relationship between the two lobes leaves the chains with the same polarity. However, the lack of sequence homology is striking. Of the 61 topologically equivalent residues there are only 10 identical residues in penicillopepsin and 14 in porcine pepsin. This may be taken to support the view that the acid protease fold has evolved convergently in the two lobes. Alternatively it indicates that the secondary and tertiary structure is conserved in evolution to a greater extent than the primary structure of a protein.

The acid protease fold described here involves the stacking of β sheets together. If divergent evolution has occurred in the two lobes, complementary changes must have occurred in residues

fusion would be selectively advantageous in assuring the correct association of dissimilar subunits. These steps are shown schematically in Fig. 6. In fact the connecting peptide between subunits is in a surface position and probably could be cleaved without denaturing the enzyme. Indeed, Rajagopalan *et al.*²⁵ have shown that porcine pepsin carrying various cleavage sites is still catalytically active and it is fascinating that this may be a reflection of the evolution of the enzyme from two smaller globular proteins.

This outline for the evolution of the acid protease structure is supported by analogous observations made in several other protein structures. Crystallographic studies of the ferredoxin from *Clostridium pasteurianum* show that the iron and labile sulphur atoms are arranged in two clusters each of which is

Fig. 5 A stereo drawing of the α carbons of the N-terminal lobe (open virtual bonds) superposed on the α carbons of the C-terminal lobe (closed virtual bonds) after rotation by the approximate twofold symmetry operation described in the text.



contributing to the core between these sheets, possibly in a protein formed by gene duplication and not under selective pressure¹⁸. Although changes may occur in one of a complementary pair of amino acid side groups which give a poorly folded or unfoldable structure, the same fold may later be optimised by further changes.

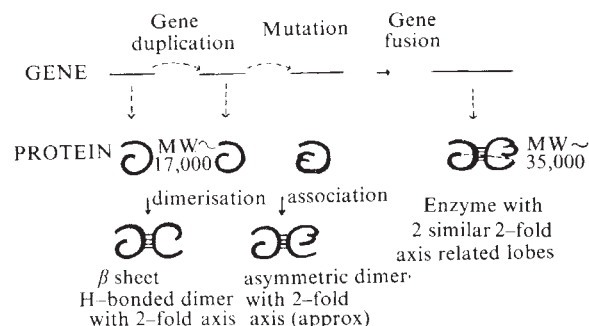
Thus it seems possible that acid proteases evolved by gene duplication of an ancestral protein of about 150 residues having a fold similar to that of one lobe of pepsin. Even before duplication a dimer of two identical subunits may have existed. The extensive hydrophobic interactions and the antiparallel pleated sheet between the two lobes of the acid protease (strands q and i) are reminiscent of similar quaternary interactions involving antiparallel sheet in insulin¹⁹ and liver alcohol dehydrogenase dimers²⁰, and in concanavalin^{21,22} and prealbumin tetramers²³. After the gene duplication event the two subunits evolved divergently so that the carboxyl groups of the two aspartyl residues developed different *pK*s and a mechanism of hydrolytic cleavage, as described recently²⁴. Finally, gene

enclosed by a similar folding of the polypeptide chain²⁶. In the bacterial ferredoxins, however, the sequences of the two similar folding units are more homologous than that which we report here for the acid protease folding domains. Rhodanese, the sulphur-transferring enzyme from bovine liver, consists of a single polypeptide chain which folds into two nearly identical domains, but only 14 of the 109 topologically equivalent residue pairs are identical in sequence²⁷. This latter figure is equivalent to the lack of homology between the two domains of the acid proteases, and indicates that a homologous amino acid sequence is not a necessary condition for similar tertiary structures. This phenomenon of folding domains that are similar in conformation but with widely differing primary structure would seem to be a rather common one.

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Fig. 6 A schematic representation of the possible evolution of the acid proteases by gene duplication.



1. Levitt, M. & Chothia, C. *Nature* **261**, 552-555 (1976).
2. Ohlsson, B. *et al. J. molec. Biol.* **89**, 339-354 (1974).
3. Rossmann, M. G. *et al. Nature* **250**, 194-199 (1974).
4. Tang, J. *Acid Proteases, Structure-Function and Biology* (Plenum, New York, 1977).
5. Hsu, I.-N. *et al. in Acid Proteases, Structure-Function and Biology* (ed. Tang, J.), 61 (Plenum, New York, 1977).
6. Hsu, I.-N. *et al. Nature* **266**, 140-145 (1977).
7. Subramanian, E. *et al. Proc. natn. Acad. Sci. U.S.A.* **74**, 556-559 (1977).
8. Jenkins, J. A. *et al. in Acid Proteases, Structure-Function and Biology* (ed. Tang, J.), 43 (Plenum, New York, 1977).
9. Fruton, J. S. *Adv. Enzym.* **44**, 1-36 (1976).
10. Tang, J. *et al. Proc. natn. Acad. Sci. U.S.A.* **70**, 3437-3439 (1973).
11. Folmann, B. & Pedersen, V. B. *in Acid Proteases, Structure-Function and Biology* (ed. Tang, J.), 3 (Plenum, New York, 1977).
12. Rossmann, M. G. & Argos, P. *J. molec. Biol.* **105**, 75-96 (1976).
13. Rossmann, M. G. & Argos, P. *J. molec. Biol.* **109**, 99-129 (1977).
14. Sepulveda *et al. J. biol. Chem.* **250**, 5082-5088 (1975).
15. Shotton, D. M. & Watson, H. C. *Nature* **225**, 811-816 (1970).
16. Delbaere, L. T. J. *et al. Nature* **257**, 758-763 (1975).
17. James, M. N. G. *Proc. Miami Winter Symp.* **11**, 125-142 (1976).
18. Altosaar, J. M. *et al. Abstracts of IUB Tenth Congress* 04-I-L-005 (1976).
19. Blundell, T. L. *et al. Nature* **231**, 506-511 (1971).
20. Eklund, H. *et al. J. molec. Biol.* **102**, 27-59 (1976).
21. Hardman, K. D. & Ainsworth, C. F. *Biochemistry* **11**, 4910-4918 (1972).
22. Edelman, G. M. *et al. Proc. natn. Acad. Sci. U.S.A.* **69**, 2580-2585 (1972).
23. Blake, C. C. F. *et al. J. molec. Biol.* **88**, 1-12 (1974).
24. James, M. N. G. *et al. Nature* **267**, 808-813 (1977).
25. Rajagopalan, T. G. *et al. J. biol. Chem.* **241**, 4940-4950 (1966).
26. Adman, E. T., Sieker, L. C. & Jensen, L. H. *J. biol. Chem.* **248**, 3987-3996 (1973).
27. Ploegman, J. H., thesis, Univ. Groningen (1977).

Purification of mouse interferon by sequential affinity chromatography on poly(U)- and antibody-agarose columns

Jaqueline De Maeyer-Guignard

Institut du Radium, Université de Paris-Sud,

M. G. Tovey and I. Gresser

Institut de Recherches Scientifiques sur le Cancer, 94800 Villejuif, France

E. De Maeyer

Institut du Radium, Bâtiment 110, Université de Paris-Sud, Orsay 91405, France

Mouse interferon has been purified to homogeneity by two-step affinity chromatography. Two polypeptide bands were obtained on sodium dodecyl sulphate-polyacrylamide gel electrophoresis migrating at molecular weights 35,000 and 22,000, both having antiviral activity. The 35,000 but not the 22,000 band, also stained with periodic acid-Schiff. The specific activity was 8×10^6 of our laboratory units, corresponding to 2.4×10^6 NIH reference units.

COMPLETE purification of interferon will make it possible to determine whether the varied biological effects attributable to interferon preparations are due to the interferon itself. Furthermore, analysis of the structure of interferon may reveal its active site(s) and eventually lead to its synthesis *in vitro*; also by labelling the molecule, it will be possible to investigate the initial interaction of interferon with the cell. Although techniques have been developed for the large-scale production¹ and purification of mouse interferon by affinity chromatography²⁻⁷, total purification has not been achieved. Knight, using gel filtration and ion-exchange chromatography, reported that purified mouse L cell interferon still had "10 to 11 polypeptide" bands after sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)⁸. We now report the purification of mouse C-243 cell interferon with a high final recovery of

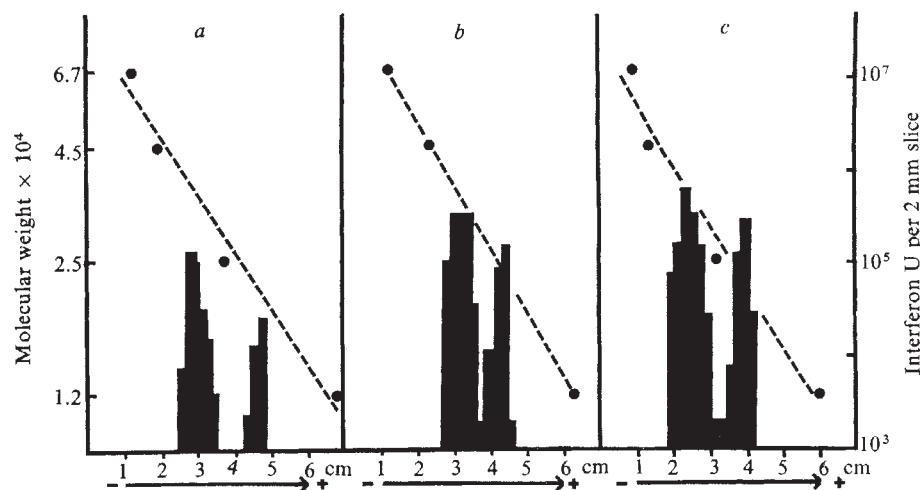
biologically active and pure material by SDS-PAGE.

Interferon titrations were performed using either a microtitre assay in L cells or a plaque reduction assay in 5-d-old secondary cultures of mouse embryo fibroblasts (MEF), with vesicular stomatitis virus (Indiana strain) as challenge virus. One microtitre unit per 0.2 ml corresponds to 10 MEF units per ml. All titres are expressed in MEF units. One MEF unit equals 0.3 NIH reference units.

Electrophoresis on acrylamide slab gels

Either plain 13% or 15% gels or exponential 10–20% gradient gels were prepared in slabs 0.075 cm thick, 11 cm long and 16 cm wide. A 5% stacking gel 1 cm high was prepared with sample wells of either 0.025 or 0.05 ml. The discontinuous buffer system using SDS has been described by Laemmli⁹. Before electrophoresis, samples were dialysed for 18 h at room temperature against 0.125 M Tris-HCl, pH 6.8, 1% SDS and 10% glycerol. β -Mercaptoethanol (1%) was added sometimes. After dialysis, some samples were concentrated 10- or 15-fold with a Minicon-B concentrator, with 15,000 molecular weight cutoff (Amicon, Lexington). Gels were run for 5–7 h depending on the concentration of the acrylamide at a constant current of 10 mA per gel, and 20 μ l of sample was usually applied per slot. After electrophoresis, gels were stained for 15 min in a solution of 0.1% Coomassie brilliant blue in a mixture

Fig. 1 Electrophoretic profile of interferon activity recovered from 10–20% acrylamide gradient gels. The starting material for (a) was 20 μ l crude interferon, concentrated tenfold, and containing 2.6×10^5 U; for (b) it was 20 μ l of interferon purified on an immunosorbent column (Fig. 2) and containing 3×10^6 U; and (c) it was 20 μ l of interferon purified on poly(U)-Sephacrose and containing 5×10^6 U. Crude interferon was used as starting material both for the chromatography on anti-interferon globulin bound to Affigel-10 and on poly(U)-Sephacrose. ---●---, Migration of molecular weight markers. For the elution of interferon from acrylamide gels, they were fractionated into 2-mm slices. Optimal results were obtained consistently when slices were eluted for at least 18 h at 4°C in electrode buffer (0.025 M Tris, 0.2 M glycine and 0.1% SDS) using 0.2 ml per slice. Total recovery of interferon activity was obtained in these conditions. The form of the gel changed as a result of staining and drying and it was not possible to correlate precisely the antiviral activity eluted from the slices obtained from a fresh gel with the stained bands of a dried preparation. This difficulty was circumvented as follows. Two aliquots of the same gel were deposited into each of two slots, with an empty slot in between. At the end of the run, before staining, the gel was cut longitudinally along a line centred on the empty slot, and care was taken not to displace the two parts of the gel with respect to each other. Another longitudinal cut, parallel to the first, was made at the other side of the sample run to be eluted; this gave a slice 1 cm wide. The latter was cut transversely every 2 mm, and each cut was prolonged a few millimetres into the gel reserved for staining. It was thus possible, after staining and drying, to correlate the antiviral activity of each fraction with protein bands by counting the notches.



of 50% methanol, 10% acetic acid and 40% distilled water; they were destained overnight in a solution of 7% acetic acid and 5% methanol in distilled water and then dried.

Crude interferon preparation

Mouse interferon was prepared from suspension cultures of Swiss mouse C-243 cells induced with Newcastle disease virus (NDV) and titred 1.3×10^6 U ml⁻¹; its protein content was 0.1 mg ml⁻¹ and its specific activity was 1.3×10^7 U per mg of protein.

The crude starting interferon preparation, when electrophoresed in the presence of SDS in non-reducing conditions, contained two peaks of activity, one migrating at 35,000 molecular weight (MW), representing about 80% of total activity, the other migrating at 22,000 MW and representing about 15% of the activity (Fig. 1a). These findings are in good agreement with those reported by Stewart *et al.* for NDV-induced L-929¹⁰ and C-243 cell¹¹ interferons (for the latter interferon they reported a molecular weight of 38,000 rather than 35,000). Likewise, we have found that treatment of interferon in reducing conditions (buffer supplemented with 1% β -mercaptoethanol) resulted in a 90% or more reduction of the antiviral activity of the 22,000 MW peak, whereas the activity of the 35,000 MW fraction was unaffected.

Interferon obtained from poly(U)-agarose and antibody-agarose

Affinity chromatography of C-243 cell interferon on poly(U)-Sephacrose 4B (Pharmacia) has been described before¹². Briefly, an interferon sample, predialysed for 24 h in 10 mM Tris-HCl, pH 7.5, is applied to the gel and desorption is obtained by adding 1 M NaCl to the Tris buffer. By this simple procedure,

Table 1 Summary of two-step purification procedure

	Volume (ml)	Titre (U ml ⁻¹)	Total U	Specific activity (U per mg of protein)
Starting material	200	1.3×10^6	2.6×10^8	1.3×10^7
Peak fractions from poly (U)-Sephacrose column	(1) 5 (2) 5 (3) 5 (4) 5	1.0×10^7 4.0×10^7 1.0×10^7 1.3×10^6	3.0×10^8 (115% of starting material)	1.2×10^9 (pool 1-4)
Peak fractions from solid phase immuno-adsorbent column	(1) 2.5 (2) 2.5 (3) 2.5 (4) 2.5 (5) 2.5	4.0×10^7 4.0×10^7 2.0×10^7 2.5×10^6 1.3×10^6		
			1.0×10^8 * (38% of starting material) 1.5×10^8 † (52% of starting material)	ND 8.0×10^9 8.0×10^9 ND ND

Crude starting material (200 ml) was dialysed overnight against 10 mM Tris-HCl buffer, pH 7.5, and was applied to a poly (U)-Sephacrose column (total bed volume 10 ml). The column was then rinsed with 10 bed volumes of Tris-HCl and desorption was carried out by adding 1 M NaCl to the buffer. The four 5-ml desorption fractions were pooled and diluted fivefold in 10 mM Tris to decrease the molarity of the preparation to be applied to the solid phase immuno-adsorbent. Thus 100 ml was applied to the anti-interferon globulin-agarose column (bed volume 4 ml). After absorption, the column was rinsed with 10 bed volumes of phosphate-buffered saline and desorption was carried out in 0.1 M citrate buffer, pH 2.2, supplemented with 1 M NaCl. Fractions (2.5 ml) were collected and the bulk of interferon activity was recovered in fractions 1-3. These fractions were concentrated 15-fold and analysed electrophoretically. Fraction 1 still contained some contaminating polypeptide bands in the low molecular weight range (<20,000) but fractions 2 and 3 showed only the two bands corresponding to interferon activity.

*This material was still slightly contaminated with other polypeptides.

†This material was electrophoretically pure. (Fig. 3a, channel 4.)

we obtained mouse interferon preparations with specific activities of up to 10^9 U per mg of protein. Furthermore, recovery was total with both peaks of activity (35,000 and

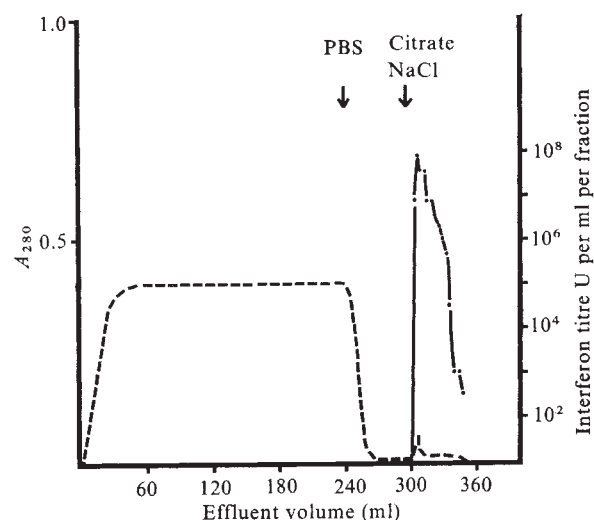


Fig. 2 Affinity chromatography of C-243 cell interferon on an anti-interferon globulin Affigel column. Anti-interferon serum was prepared and partially purified as before¹³. The partially purified anti-mouse interferon serum used had a neutralising titre of 2.4×10^8 U when assayed against 16 U of interferon. One ml of serum neutralised 6.4×10^8 interferon reference units. Immunoglobulins were precipitated with 2.1 M ammonium sulphate, processed as before² and coupled to an agarose matrix with a spacer arm, Affigel-10 (Bio-Rad). The globulin fraction obtained from 15 ml of the anti-interferon serum was fixed to 1 g of Affigel-10. A crude interferon preparation (240 ml) containing a total of 3.12×10^8 U was applied to the column. The loaded column was washed with 10 bed volumes of phosphate-buffered saline (60 ml) and desorbed with citrate buffer (1 M NaCl, 0.1 M Na citrate, pH 2.2); 3.0-ml fractions were collected. Total recovery was 3.7×10^8 U, that is 119% of input, and the specific activity of the pooled peak fractions was 2×10^9 U per mg of protein. An aliquot of the peak fraction with the highest titre was dialysed against sample buffer, concentrated 10-fold, and analysed on a 15% acrylamide gel (Fig. 3a, channel 3). Protein content was determined by the fluorometric assay described by Boehlen *et al.*¹⁴. In some instances the protein content was estimated after PAGE from the intensity of the staining of the bands with Coomassie brilliant blue. This was done by comparison with bands obtained after electrophoresis of known amounts of bovine serum albumin and ovalbumin. — — —, Absorbance at 260 nm; —●—, interferon titre.

22,000 MW) being recovered (Fig. 1c). As Fig. 3a shows (channel 2), the two bands corresponding to interferon can be seen after staining. In addition to the two polypeptide bands corresponding to interferon, there are many other contaminating polypeptides.

Purification of C-243 cell interferon on antibodies covalently bound to agarose through a spacer arm (Affigel-10) is described in Fig. 2 and the product is illustrated in Fig. 3. Interferon recovery was complete and, on electrophoresis a migration pattern comparable with that of the starting material was obtained (Fig. 1b). Again, although an extensive degree of purification was achieved (230-fold to yield a product with specific activity about 3×10^9 U per mg of protein), contaminating polypeptides were still present in the preparation (Fig. 3a, channel 3). Because the polypeptide contaminants retained by the affinity chromatography of the crude interferon preparation on poly (U)-agarose were different from those retained by the antibody-agarose column, we decided to combine the two techniques.

Sequential affinity chromatography

The crude interferon preparation was first purified on poly (U)-Sephacrose and the product was applied to an anti-interferon globulin column. Experimental details and results are given in Table 1 and illustrated in Fig. 3*a*, channel 4. After concentration and electrophoresis of an aliquot of fractions 2 or 3,

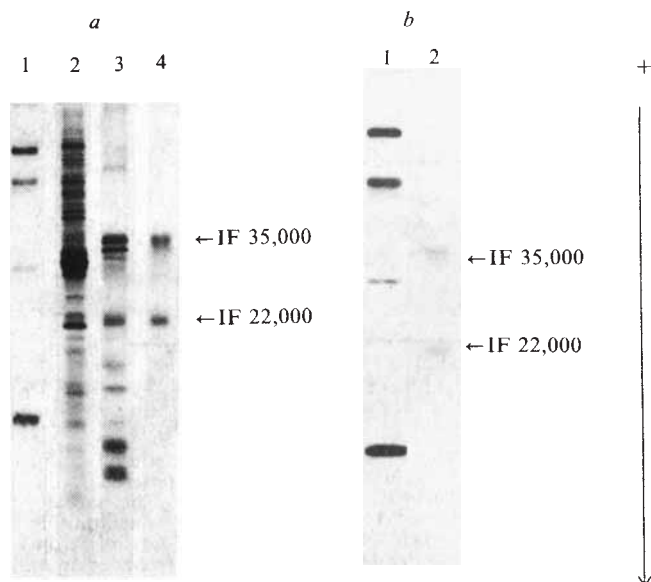


Fig. 3 SDS-PAGE in a 15% gel of interferon (IF) preparations of different degrees of purity. *a*, Input per channel was 20 μ l in each case. Channel 1, molecular weight markers; channel 2, after purification on poly (U)-agarose; input was 1×10^7 U; channel 3, after purification on antibody-agarose; input was 1.3×10^7 U; channel 4, after combination of the two methods; input was 1.2×10^7 U. *b*, Migration of interferon after boiling in denaturing conditions. Channel 1, molecular weight markers; channel 2, purified preparation containing 6×10^6 U in 10 μ l. Molecular weight markers from top to bottom: bovine serum albumin, ovalbumin, chymotrypsinogen and cytochrome *c*.

only two protein bands migrating at 35,000 and 22,000 MW were observed. When slices of the uncoloured gel corresponding to these two bands were eluted for biological activity, there was excellent correlation between interferon activity and the stained bands (Fig. 4). As Fig. 3*a*, channel 4, and Fig. 3*b*, channel 2 show, this preparation can be considered pure because no other bands were detected (the limits of detection with the method in our hands was 0.07 μ g of either bovine serum albumin (BSA) or ovalbumin). In one experiment we put 2.5 times as much material on the gel as was used for Fig. 3*a*, channel 4, by starting the electrophoresis with 20 μ l and refocusing twice, each time with 15 μ l (using a higher stacking gel). The two interferon bands were more diffuse than those obtained in normal conditions, which was expected in view of the way the material was placed on the gel, but again no contaminating bands were observed. In addition to electrophoresis on a 15% gel, the pure material was run in a 13% gel and in a 10–20% gradient gel, and each time antiviral activity was present in gel slices corresponding to the two stained bands.

For the interferon band to be quite distinct, it was necessary to put a minimum of 20 μ l per slot of a preparation of titre 3×10^8 U ml⁻¹ (that is 6×10^6 U per slot). In these conditions the intensity of the stain with Coomassie brilliant blue corresponded roughly to 0.75 μ g of protein, for the two bands combined, using BSA as standard. This represents 37.5 μ g of

interferon per ml for a preparation of titre 3×10^8 U ml⁻¹, which means that the specific activity was 8×10^9 U per mg of protein. The 35,000 MW band was rather broad, but such dispersion is not unusual for glycoproteins and there is evidence that at least some interferons are glycoproteins^{15,16}.

To determine whether the purified product contained sugar we stained the gel with periodic acid-Schiff (PAS) after PAGE in a 15% gel. We used 15 μ l of the same concentrated preparation used to stain the polypeptide bands with Coomassie brilliant blue as shown in Fig. 3. While the 35,000 band stained almost as intensely with Schiff's reagent as with Coomassie brilliant blue, the 22,000 band hardly stained at all. No other bands were visible. Thus there was more sugar present in the 35,000 MW fraction than in the 22,000 MW fraction.

To exclude the possibility that the 35,000 MW component was a dimer of the 22,000 MW component, an aliquot of the preparation that had been electrophoresed for activity (Fig. 3*a*, channel 4 and Fig. 4) was supplemented with 1% β -mercaptoethanol and boiled for 1.5 min before electrophoresis. This treatment did not affect migration of either band (Fig. 3*b*, channel 2).

Correlation between antiviral and cell multiplication inhibitory activities

Individual fractions, after SDS-PAGE, of the 35,000 band purified interferon were tested for antiviral activity on L cells

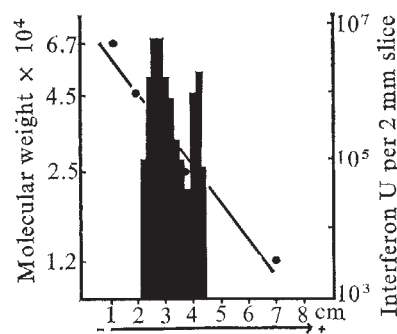


Fig. 4 Profile of interferon activity recovered after electrophoresis of the pure preparation. An aliquot of fraction 2 obtained after desorption of the anti-interferon globulin-agarose column (Table 1) was dialysed and concentrated 15-fold before electrophoresis (see legend to Fig. 1). A 20- μ l sample was electrophoresed in a 15% acrylamide gel for staining (Fig. 3), and in an adjacent channel another 20- μ l sample was electrophoresed to recover activity. The theoretical input was 1.2×10^7 U per channel. The total recovery of interferon activity after electrophoresis was 1.9×10^7 U (158% of input); 79% of this activity was recovered from the three slices corresponding to the 6-mm area of the stained 35,000 molecular weight band, and 19% from the two slices corresponding to the 4-mm area of the 22,000 band. The remaining 6% was recovered from the area in between and adjacent to the bands. Comparable results were obtained when the electrophoresis was carried out either in a 13% or in a 10–20% acrylamide gradient gel. —●—, Migration of molecular weight markers.

and cell multiplication inhibitory activity on murine leukaemia L1210 cells, as described before^{11,17}. As Fig. 5 shows, there was an excellent quantitative correlation between the two activities.

We conclude therefore that mouse interferon can be purified considerably by affinity chromatography using poly (U)-Sephacrose¹² or anti-interferon globulin columns². Combining both techniques, we obtained interferon that seemed pure after SDS-PAGE—we consistently obtained only two bands after staining either with Coomassie brilliant blue or PAS.

The fact that both the 35,000 and 22,000 MW components were purified together on the antibody-agarose column does not imply that they are antigenically identical, for immunisation had been carried out with the crude starting material containing both components. They were also purified together on the poly (U)-agarose column, indicating that each of them has a polynucleotide attachment site¹². By comparing the intensity of coloration of each band with a known BSA standard, we estimate that this interferon has a specific activity of at least 8×10^9 U (2.4×10^9 NIH reference units).

Our results differ in several important respects from those obtained by Knight who reported the purification of mouse interferon from L cells induced with MM virus⁸. His purified interferon preparation contained 10–11 different bands, whereas ours contained only two bands when stained with

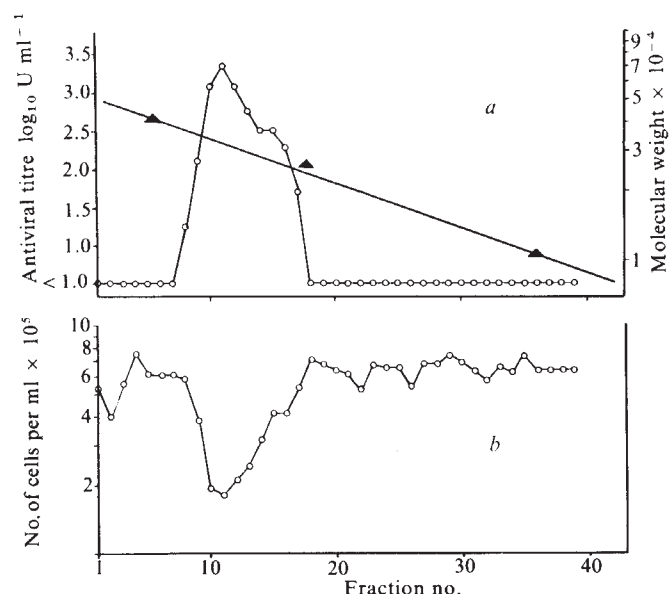


Fig. 5 Anticellular activity after elution of pure interferon from a 13% gel (with 1% β -mercaptoethanol). *a*, Antiviral activity as assayed on mouse L cells challenged with vesicular stomatitis virus. *b*, Cell multiplication inhibitory activity as assayed on mouse leukaemia L1210 cells^{11,17}. All gel eluates were incubated with 6×10^4 L1210 cells per ml in 2 ml of RPMI 1640 medium containing 10% foetal calf serum, and cell multiplication was determined after 3 d of incubation by counting viable cells in a haemocytometer and using the Trypan blue dye exclusion test. All eluates were tested at a 1:10 dilution. All points are averages of duplicate determinations. $\blacktriangle$, Migration of molecular weight markers: ovalbumin, chymotrypsinogen and cytochrome *c*.

Coomassie brilliant blue or PAS, and the interferon peaks were more pronounced and with much less dispersion of biological activity than in his study. Our 22,000 MW peak represented only 15% of total activity and stained less with PAS than did the 35,000 MW peak, in contrast to Knight's results in which the low molecular weight peak was the major component and also stained more intensely with PAS. The possibility that we have selected interferon molecules at both extremes of the molecular weight range during purification, and lost the intermediate ones, can be ruled out, because recovery from chromatography on both antibody and poly (U) was close to 100%, and in addition we showed that the interferon activity of the crude starting material had an electrophoretic profile identical to that of the purified product. These differences between Knight's results⁸ and those reported here may stem from either a greater degree of purity of our final product or differences in the characteristics of the interferon produced in the two virus-cell systems.

Our results suggest, in agreement with Knight's findings⁸, that the 35,000 MW component is not a dimer of the 22,000 MW component because the electrophoretic profile did not change after boiling in denaturing conditions. Possibly the two components are different gene products, and there are some data to suggest this may be so for human leukocyte interferon¹⁸. If the two polypeptides were different gene products, the proportion in which they are produced could well depend on the cell and also on the inducer used. It is also possible that the 22,000 MW component corresponds to the polypeptide part of the 35,000 MW component from which the sugar moiety has been removed. In addition, the low biological activity found between the two stained bands may correspond to the interferon peptide with intermediate degrees of glycosylation.

We have presented evidence that interferon can inhibit cell multiplication^{11,17} and have suggested that interferon has an important role in the regulation of cell division and function¹⁹. Although this has been a subject of some controversy (see ref. 11 for references), the results presented here show that there is excellent correlation between the antiviral and cell multiplication inhibitory activities of our pure mouse interferon. Knight⁸ has reported that purified human fibroblast interferon also inhibited the multiplication of human fibroblasts. Now that mouse and human interferon have been purified it will be important to determine whether other biological effects (see refs in ref. 19) observed with crude or semipurified preparations are due to interferon.

The specific activity of purified mouse C-243 cell interferon is 2.4×10^9 NIH reference units per mg of protein, which means that for a molecular weight of 22,000 interferon is active at a concentration of 1.9×10^{-15} molar. Interferon would thus seem to be one of the most biologically active of all polypeptides.

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1. Tovey, M. G., Begon-Lours, J. & Gresser, I. *Proc. Soc. Exp. Biol. Med.* **146**, 809–815 (1974).
2. Sipe, J. D., De Maeyer-Guignard, J., Fauconner, B. & De Maeyer, E. *Proc. natn. Acad. Sci. U.S.A.* **70**, 1037–1040 (1973).
3. Ogburn, C. A., Berg, K. & Paucker, K. *J. Immun.* **111**, 1206–1218 (1973).
4. Anfinsen, C. B., Bose, S., Corley, L. & Gurari-Rotman, D. *Proc. natn. Acad. Sci. U.S.A.* **71**, 3139–3142 (1974).
5. Davey, M. W., Sulikowski, E. & Carter, W. A. *J. Virol.* **17**, 439–445 (1976).
6. Davey, M. W., Sulikowski, E. & Carter, W. A. *J. biol. Chem.* **251**, 7620–7625 (1976).
7. De Maeyer-Guignard, J. & De Maeyer, E. *C.r.hebd. Seanc. Acad. Sci.* **D283**, 709–711 (1976).
8. Knight, E. Jr *J. biol. Chem.* **250**, 4139–4144 (1975).
9. Laemmli, U. K. *Nature* **227**, 680–685 (1970).
10. Stewart, W. E. II *Virology* **61**, 80–86 (1974).
11. Stewart, W. E. II, Gresser, I., Tovey, M. G., Bandu, M. T. & Le Goff, S. *Nature* **262**, 300–302 (1976).
12. De Maeyer-Guignard, J., Thang, M. N. & De Maeyer, E. *Proc. natn. Acad. Sci. U.S.A.* **74**, 3787–3790 (1977).
13. Gresser, I., Tovey, M. G., Bandu, M. T., Maury, C. & Brouty-Boyé, D. *J. exp. Med.* **144**, 1305–1315 (1976).
14. Boehlen, P., Stein, S., Dairman, W. & Udenfriend, S. *Arch. Biochem. Biophys.* **155**, 213–220 (1973).
15. Dörner, F., Scriba, M. & Weil, R. *Proc. natn. Acad. Sci. U.S.A.* **70**, 1981–1985 (1973).
16. Havell, E. A., Yamazaki, S. & Vilček, J. *J. biol. Chem.* **252**, 4425–4427 (1977).
17. Gresser, I., Brouty-Boyé, D., Thomas, M. T. & Macieira-Coelho, A. *Proc. natn. Acad. Sci. U.S.A.* **66**, 1052–1058 (1970).
18. Cavaliere, R. L., Havell, E. A., Vilček, J. & Pestka, S. *Proc. natn. Acad. Sci. U.S.A.* **74**, 3287–3291 (1977).
19. Lindahl, P., Leary, P. & Gresser, I. *Proc. natn. Acad. Sci. U.S.A.* **69**, 721–725 (1972).

Structure of pyruvate kinase and similarities with other enzymes: possible implications for protein taxonomy and evolution

Michael Levine*, Hilary Muirhead†, David K. Stammers‡ & David I. Stuart

Molecular Enzymology Laboratory, Department of Biochemistry, University of Bristol, Bristol, UK

The structure determination of pyruvate kinase shows that each subunit of the tetrameric molecule consists of three domains. The largest of these domains has a remarkable similarity to the structure of triosephosphate isomerase. Another domain shows similarities to many other nucleotide binding proteins. We discuss these similarities and their implications for current arguments on protein taxonomy and evolution.

WE present here results from our X-ray determination of the three-dimensional structure of cat muscle pyruvate kinase (PK) at 2.6 Å resolution which may bear on questions of protein taxonomy and evolution. The X-ray data were collected photographically using an Arndt-Wonacott rotation camera¹, and phase angles were estimated by the method of isomorphous replacement with anomalous scattering measurements using three derivatives. Full experimental details will be published elsewhere.

The high resolution structures are known for about 50 proteins and certain patterns of structural similarity have appeared in a large proportion of these. Their close correlation with functional similarities has led some workers to propose evolutionary schemes for these proteins^{2,3}, although others have suggested that there are only a limited number of stable structures from which proteins are constructed^{4,5}. In the globins and some serine proteases close correspondence in tertiary and primary structure, as well as function, strongly suggests divergence from an ancestral gene^{2,3}. Amongst the dehydrogenases the sequences differ, but the dinucleotide binding domain forms a common structural feature^{6,7} and divergence has been suggested here too. The observation that the 'mononucleotide binding fold' (mnbf) defined as one half of the dehydrogenase NAD binding domain, namely three parallel strands of β sheet and two interconnecting α helices (see Fig. 2e), is common to many other nucleotide binding proteins led Eventoff and Rossmann to propose that this may be an important evolutionarily conserved unit in the kinases⁸. (We shall use the term mnbf to designate this structure without implying any binding properties).

Our studies on PK have shown that the part of the structure involved in the binding of substrates and cofactors bears a striking similarity to the structure of triosephosphate isomerase (TIM)^{9,10}, an enzyme which does not require nucleotides, and is quite unlike the known structures of the other kinases¹¹⁻¹⁴. Another part of the molecule resembles the mnbf and nucleotides bind here but in a novel fashion.

Structure of pyruvate kinase

Details of the structure of one subunit of the PK molecule are shown in Fig. 1. Each subunit comprises some 500 amino acid residues and the molecular weight of the complete molecule, consisting of four identical subunits related by twofold axes as shown, is about 240,000. The polypeptide chain is folded into three distinct domains which are shown schematically in Fig. 1. Domain A is the largest, composed of about 220 residues, and contains a cylindrical β sheet of eight parallel strands. Adjacent strands are connected by α helices which form an outer cylinder coaxial with the first. This is shown in stereo in Fig. 2a and differs from the interpretation of the 3.1-Å map in that an extra strand of β sheet has been located¹⁵. This folding pattern has been observed previously in TIM⁹. To compare these two structures a computer search procedure similar to that of Rossmann and Argos¹⁶ was carried out (Table 1 columns 5-10). A detailed comparison of the arrangement of β sheet and α helix in the two structures is shown in Fig. 2a-d. To represent this on a two dimensional surface the α -carbon coordinates have been expressed in cylindrical polar form thereby effectively 'unwrapping' the barrel (Fig. 2c, d). Between the third strand of β sheet and third helix of domain A the chain folds up into domain B, where about 100 residues have been located so far. As can be seen in Fig. 2, domain B occurs in the same position as the largest loop in TIM. Domain B does not seem to consist of α/β structure¹⁷, the main secondary structural feature being anti-parallel β sheet. In TIM the chain terminates at the end of the final helix of the barrel whereas in PK the chain continues for about another 120 residues forming domain C. The first section of domain C comprises two long anti-parallel α helices. The rest of the chain is folded up into a five-stranded β sheet flanked by α helices. The first three strands of sheet and two inter-connecting α helices form the well known mnbf. The third helix leads to the fourth strand of sheet and the last strand lies between strands 1 and 4 and anti-parallel to them. The remarkable similarity of part of domain C to the first mnbf of lactate dehydrogenase (LDH) is illustrated in Fig. 2e.

Comparisons of structure and function

The comparisons of these folding patterns were quantified using the computer search procedure referred to above and the results are given in Table 1 columns 5-10. This shows that the similarity in fold between TIM and domain A of PK is more significant than that between the dehydrogenases. A part of domain C agrees as well with the mnbf of LDH as does phosphoglycerate kinase (PGK), but, the mnbf's of the dehydrogenases LDH and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) agree rather better amongst themselves.

Substrate and cofactor difference maps have been published at 6-Å resolution previously for PK¹⁸. Phosphoenolpyruvate (PEP) lies at the carboxyl end of the barrel close to its axis and interacts with several large side chains. The

*Present address: Department of Physiology, University of Bristol, Bristol, UK.

†To whom reprint requests should be addressed.

‡Present address: The Wellcome Research Laboratories, Beckenham, Kent, UK.

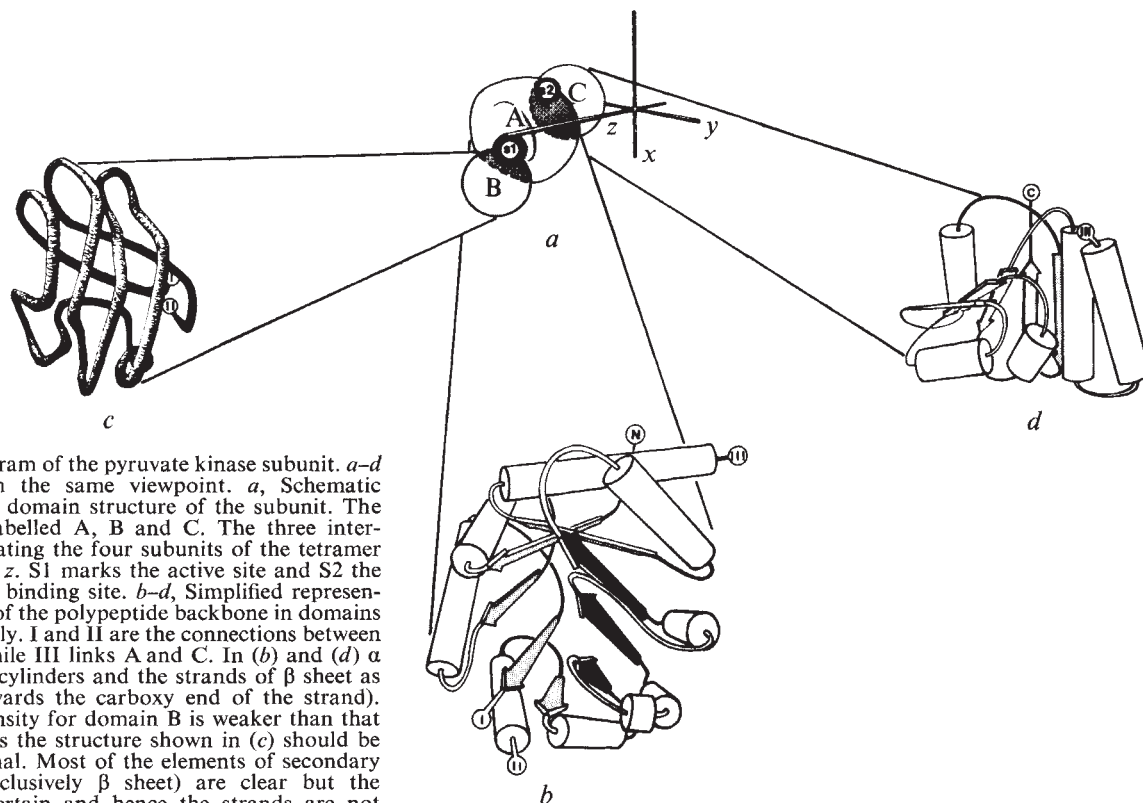


Fig. 1 Exploded diagram of the pyruvate kinase subunit. *a-d* Are all drawn from the same viewpoint. *a*, Schematic representation of the domain structure of the subunit. The three domains are labelled A, B and C. The three intersecting dyad axes relating the four subunits of the tetramer are labelled *x*, *y* and *z*. S1 marks the active site and S2 the secondary nucleotide binding site. *b-d*, Simplified representations of the course of the polypeptide backbone in domains A, B and C respectively. I and II are the connections between domains A and B while III links A and C. In (*b*) and (*d*) α helices are shown as cylinders and the strands of β sheet as arrows (pointing towards the carboxy end of the strand). Since the electron density for domain B is weaker than that for the other domains the structure shown in (*c*) should be regarded as provisional. Most of the elements of secondary structure (almost exclusively β sheet) are clear but the connections are uncertain and hence the strands are not shown as arrows.

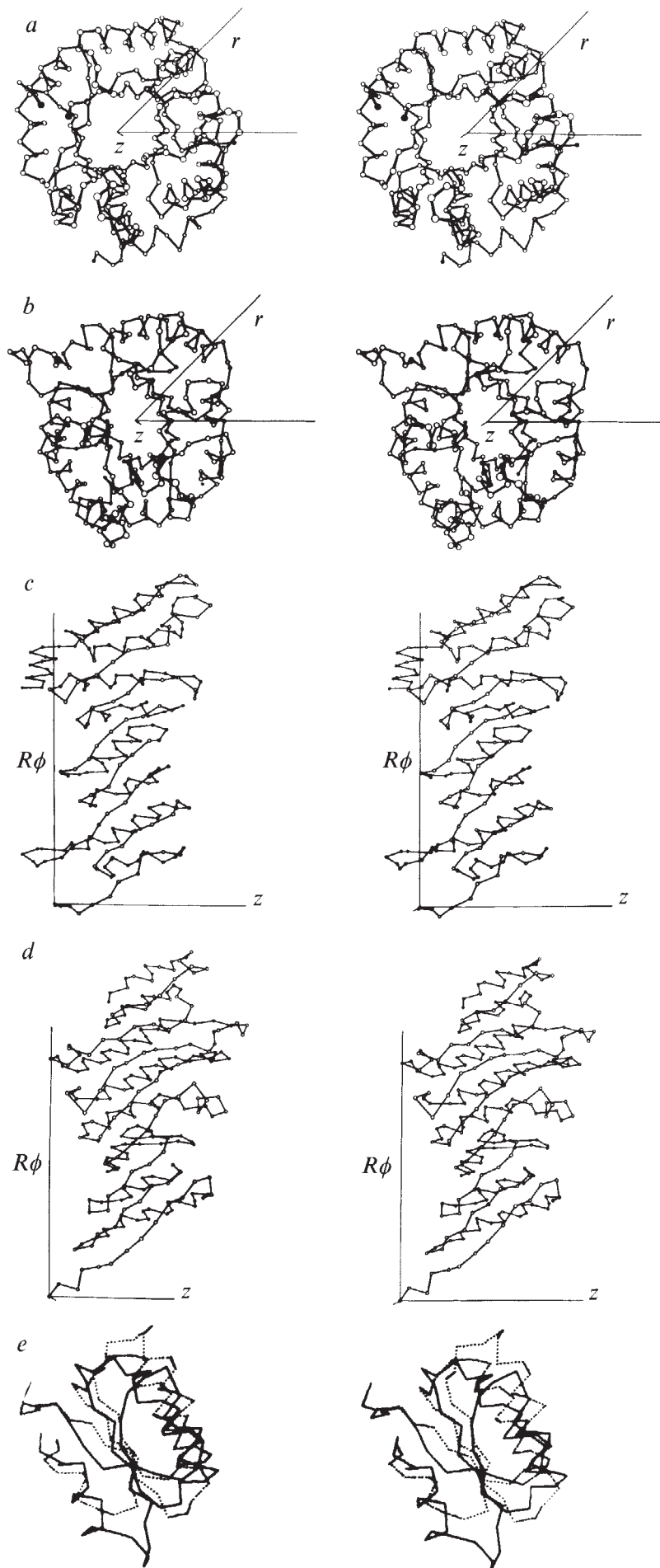
Table 1 Results of structural comparisons between domains A and C of PK and other enzymes

(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
Structure 1	Structure 2	Figure of topological relatedness		No. of equivalent residues between 1 and 2	E_1	E_2	% of equivalent residues in structure 1	% of equivalent residues in structure 2	r.m.s. deviation for these equivalents (Å)
PK domain A	TIM	2,580,480	5,040	160	2.7	6.0	160/216 (74%)	160/247 (65%)	3.0
LDH dinucleotide binding fold	GAPDH	11,520	442	83	2.7	6.0	83/164 (51%)	83/162 (51%)	3.1
PK domain C mnbf	LDH	12	3	54	2.4	6.0	54/ 67 (81%)	54/ 76 (71%)	2.7
PGK mnbf	LDH	12	3	58	2.4	6.0	58/128 (45%)	58/ 76 (76%)	2.9
GAPDH mnbf	LDH	12	3	59	2.4	6.0	59/ 90 (66%)	59/ 76 (78%)	2.1

Columns (3) and (4): these measures are limited by being based on the extant structures and do not consider the significance that, for instance, 2 structures are eight-stranded α/β barrels, whatever the connections. However, since this measure has been used before in assessing the significance of structural similarities the numbers allow our results to be related to previous discussions, on the basis of simple, explicit assumptions. Col. (3), Schulz & Schirmer²² calculated the number of distinct topologies which could be obtained by re-connecting the helices and β sheet strands of a given α/β structure. In their treatment the 'topology' is fully described by strand sequence in the sheet and the above pattern of connections. For n strands of sheet there are $n!$ arrangements and 2^{n-1} ways of arranging the interconnecting helices above or below the sheet. Of these topologies half are superimposable on the other half by twofold rotation. Thus there are $n! \times 2^{n-2}$ distinct topologies. Col. (4), Sternberg and Thornton⁵, and Richardson⁴ have pointed out that sheet and helix in α/β structure are nearly always connected in such a way as to form a right-handed spiral. This reduces the number to approximately $n!/2$. For an eightfold barrel, assuming that the helices, for steric reasons, cannot be on the inside, we can choose the first strand anywhere. There are then seven choices for the second and so on. There are thus $(n-1)!$ distinct topologies. Since the barrel is a closed structure the restriction of Sternberg and Thornton does not affect this total. Cols (5)–(10), structure 2 is rotated through all possible angles and compared to structure 1 for each position (Rossmann and Argos¹⁶). The likelihood that residue i in structure 1 is equivalent to residue j in structure 2 is expressed as the probability $P_{ij} = \exp(d_{ij}^2/E_1^2) \exp(S_{ij}^2/E_2^2)$. d_{ij} = the distance between $c_{a(i)}$ and $c_{a(j)}$ in the current orientations of the molecules $S_{ij}^2 = (d_{ij} - d_{i+1,j+1})^2 + (d_{ij} - d_{i-1,j-1})^2$ and gives a measure of how similar the shapes of the chains are on either side of $c_{a(i)}$ and $c_{a(j)}$. By varying the values of E_1 and E_2 we can adjust the relative weights attached to each of these two measures in assessing the overall similarity of two stretches of polypeptide chain. P_{ij} is calculated for all values of i and j (ref. 23). We choose that set of (i,j) for which the sum $\sum P_{ij}$ ($P > 0.05$) is maximum and i and j both increase from one pair to the next along the chains. Equivalent residues are then those residues which have $P_{ij} > 0.05$, the physical significance of this cut off being determined by the particular values that are used for E_1 and E_2 . Orientation and translation parameters are found for which the number of equivalences is largest²⁴. These numbers are in Col. (5) and expressed as percentages of each structure in Col. (8) and (9). The rotation matrix which brings TIM (protein data bank coordinates) into the same orientation as PK is:

$$\begin{array}{ccc} -0.7376 & 0.5956 & -0.3182 \\ -0.5214 & -0.2029 & 0.8288 \\ 0.4291 & 0.7773 & 0.4602 \end{array}$$

Fig. 2 Stereo diagrams showing the results of structural comparisons of pyruvate kinase with other enzymes. The α -carbon backbone is represented. The pyruvate kinase coordinates are as measured from a 1 cm/ $\text{\AA}$ skeletal model. *a*, Domain A of PK. *b*, TIM, rotated so as to give the overall 'best fit' as defined in Table I when superimposed on domain A of PK. Note that the cross section of the barrel is more elliptical than in PK. *c*, Domain A of PK unwrapped. The cartesian coordinates of the α carbons have been transformed into cylindrical polar coordinates r , z and Φ where the cylindrical polar axes are as shown in (*a*). r is the distance along the azimuthal vector, Φ is the azimuthal angle and z is the distance along the cylinder axis. R is the approximate radius of the β sheet cylinder. $R = 8.5 \text{ \AA}$. *d*, TIM unwrapped as in (*c*). $R = 8.0 \text{ \AA}$. This shows clearly any small differences in the orientation of the structure elements. *e*, The mnbf LDH (dotted line) compared to part of domain C of PK (full line).



density for Mn-ATP lies radially perpendicular to the barrel axis so that the end of the density interpreted as the terminal phosphate overlaps the PEP binding site and the other end is between strands 3 and 4 of the barrel β sheet. This is unlike PGK where the 3-phosphoglycerate (N. Walker, personal communication) and nucleotide^{11,12} lie along the end of the β sheet. However, the PEP density is in a similar position to that of the substrate dihydroxyacetone phosphate (DHAP) in TIM⁹ (see Fig. 4). In suitable conditions ADP can be bound at a site between domains A and C in such a position that it interacts with the amino ends of β strands of the barrel and the carboxy end of the second helix of the mnbf in domain C. Surprisingly it is not at the carboxy end of the β sheet of this structure (Fig. 1).

Since kinases are grouped together on the basis of phosphoryl transfer from ATP the similarity between domain A of PK and TIM is at first sight unexpected. However, there are in fact similarities in the reactions of the two enzymes if we consider enolisation of the 2-keto-triose substrates. In PK, PEP is produced by deprotonating and phosphorylating pyruvate, while in TIM the formation of glyceraldehyde-3-phosphate is accomplished through deprotonation of dihydroxyacetone phosphate. It has been proposed that in each case a base on the enzyme participates in the deprotonation reactions. The existence of a *cis*-enediol intermediate has been demonstrated for TIM^{19,20}. Rose has shown that in the reaction catalysed by PK, enolisation of pyruvate may be decoupled from phosphorylation²¹. Figure 3 illustrates the similarity in this part of the proposed mechanisms.

We have already noted that the substrate binding sites are similarly located on the axis of the barrel in both PK and TIM. The next question to ask is are the active sites of the two proteins similar? Since the primary structure of PK is unknown we cannot identify the side chains with any certainty. We present the side chains which appear in the electron density map in the region where substrates bind, together with the corresponding region of the TIM model (Fig. 4). From this diagram it seems that there are side chains in the active sites of both PK and TIM that are associated with corresponding β -sheet elements. As far as can be seen the sizes and orientations are similar. Thus, as well as comparing PK with the other kinases, it may be useful to include consideration of the fine differences between PK and TIM in studies of the mechanisms of these two enzymes.

Fig. 3 The proton transfer reactions catalysed by pyruvate kinase and triose phosphate isomerase. In each case B is a base on the enzyme. Enol-pyruvate can be considered to be an enzyme-bound intermediate in the PK reaction²⁶ and a *cis*-enediol intermediate has been implicated in the TIM reaction²⁰.

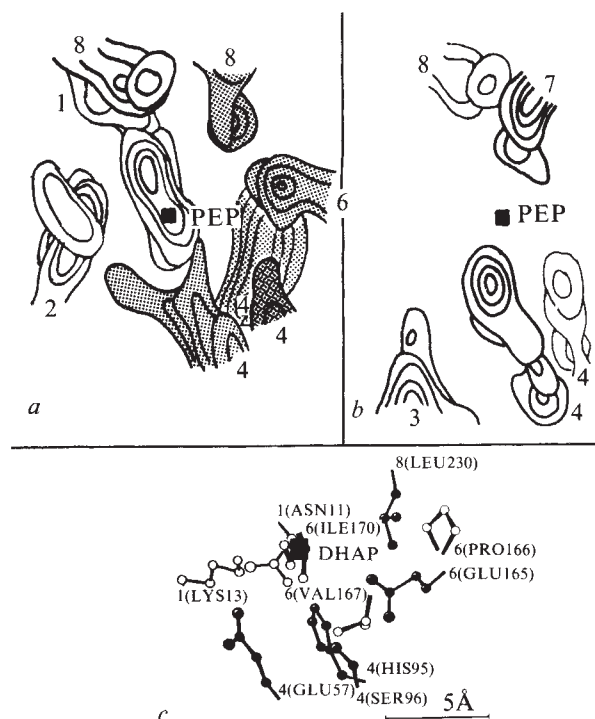
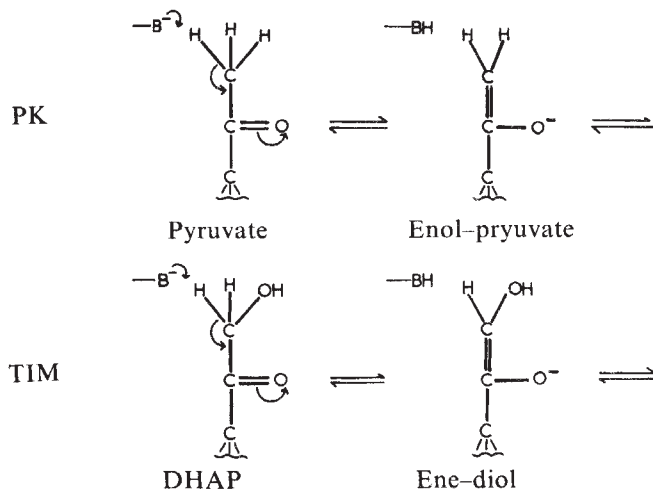


Fig. 4 *a, b*, Pyruvate kinase active site. The electron density corresponding to side chains close to the binding site of PEP is shown, this is the area marked S1 in Fig. 1. The direction of view is along the axis marked y in Fig. 1 with the z axis horizontal. Each side chain is numbered to indicate which element of the β -sheet barrel it is attached to. The n th element of the barrel is defined as containing all residues between the beginning of the n th strand and the end of the n th helix. In the electron density map, the unit cell of the crystal is sectioned perpendicular to the y -axis. The thickness of each section is 1.25 Å. The PEP site is centred on section 38 and is marked ■. (*a*) Shows a composite of sections 32 to 38 and *b*, of sections 38 to 42. The side chains only are shown, the main chain having been left out for clarity. *c*, Triosephosphate isomerase active site. The orientation corresponds to that of (*a*) and (*b*) and the atomic coordinates for the side chains of the residues close to the DHAP position are shown. C_α of DHAP is marked ■. The labels show the element of barrel to which each residue is attached with the residue type and number given in parenthesis. The scale of this diagram is the same as (*a*) and (*b*). The residues shaded in (*a*) seem to occupy analogous positions to those with filled atoms in (*c*) and it will be seen that the secondary structural elements that they are attached to correspond. Phillips *et al.*²⁶ have shown that the proposal, based on the X-ray diffraction results, that Glu 165 is the base implicated in the mechanism (Fig. 3) is consistent with other biochemical evidence. In comparing the two diagrams note that the barrels are not exactly the same shape in TIM and PK (Fig. 2*a, b*) and consequently groups attached to different strands are displaced relative to each other. Note also that three of the residues of the fourth sheet-helix element seem to be in similar relative positions in the two structures. These three residues in TIM²⁶, His 95, Ser 96, Glu 97, are all members of the group of residues implicated in the active site.

Pyruvate kinase and evolution

Our results suggest that domain A of PK and TIM may have evolved from a common ancestor but contradict the idea³ that the appearance of the mnbf in an enzyme implies the binding of a nucleotide in the same fashion as in the dehydrogenases and its evolutionary conservation for this function alone. Thus if one considers that any three consecutive strands of the eightfold barrel comprise a mnbf⁹ then the two nucleotide binding sites in PK are both associated with a mnbf. However, the manner of binding is quite different to that found in the dehydrogenases. At the active site the nucleotide is bound at the carboxy end of the β sheet as in the dehydrogenases but is orientated along a radius instead of along the edge of the sheet. At the secondary site it is not even near to the β sheet. It could be

argued^{4,5} that a structure like the mnbf is likely to form in PK if only because it is so highly probable in such a large α/β structure. We feel that although this may be true it tends to divert attention from interesting considerations of structure and function and their possible relation to evolution. It may be that proposals for simple evolutionary relationships between structures should at present be confined to functional domains as in the dehydrogenases or domain A of PK and not applied to stable substructures such as the mnbf which could conceivably be dissected out from them. If the similarities between domain A and TIM do imply a divergent evolutionary relationship rather than convergent evolution then it would seem that either PK is a composite of parts which had separate evolutionary histories before their joining together to make the modern enzyme or TIM is derived from a fragment of an ancestral protein. We would still have to account for the derivations of domains B and C in PK. It is also apparent that the kinases do not form such a closely related group of structures as the dehydrogenases. All known kinase structures consist of α/β units¹¹⁻¹⁴. PGK^{11,12} possesses the 'dinucleotide binding fold' of LDH and apparently binds the nucleotide in a similar manner. PK, hexokinase¹⁴ and adenyl kinase¹³ all differ.

The basic difference between the PK/TIM homology and those observed previously is that whereas for, say, the dehydrogenases the function of the postulated conserved gene is characteristic of a group of enzymes accepted as belonging to a single class, in the PK/TIM case, the function conserved would cut across the two categories 'kinase' and 'isomerase'. As more protein structures are revealed the present categories may therefore be found

inadequate and other groupings may emerge which reflect both similarities of domain structure and the different aspects of the function. The problem posed by the structural diversity of the enzymes now classed as kinases may then disappear.

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1. Arndt, U. W., Champness, J. N., Phizackerley, R. P. & Wonacott, A. J. *J. appl. Crystallogr.* **6**, 457-463 (1973).
2. Dayhoff, M. O., Hunt, L. T., McLaughlin, P. J. & Jones, D. D. *Atlas of Protein Sequence and Structure* **5**, 17-30 (1972).
3. Hartley, B. S. *Phil. Trans. R. Soc. B* **257**, 77-78 (1970).
4. Richardson, J. S. *Proc. natn. Acad. Sci., U.S.A.* **73**, 2619-2623 (1976).
5. Sternberg, M. J. E. & Thornton, J. M. *J. molec. Biol.* **105**, 367-382 (1976).
6. Rossmann, M. G., Moras, D. & Olsen, K. W. *Nature* **250**, 194-199 (1974).
7. Ohlsson, I., Nordström, B. & Brändén, C.-I. *J. molec. Biol.* **89**, 339-354 (1974).
8. Eventoff, W. & Rossmann, M. G. in *CRC Critical Reviews of Biochemistry* (ed. Fasman, G. D.), vol. 3, 111-140 (CRC Press, Cleveland, 1975).
9. Banner, D. W. *et al. Nature* **255**, 609-614 (1975).
10. Banner, D. W., Bloomer, A. C., Petsko, G. A., Phillips, D. C. & Wilson, I. A. *Biochem. biophys. Res. Commun.* **72**, 146-155 (1976).
11. Blake, C. C. F. & Evans, P. R. *J. molec. Biol.* **84**, 585-601 (1974).
12. Bryant, T. N., Watson, H. C. & Wendell, P. L. *Nature* **247**, 14-17 (1974).
13. Schulz, G. E., Elzinga, M., Marx, F. & Schirmer, R. H. *Nature* **250**, 120-123 (1974).
14. Steitz, T. A., Fletterick, R. J., Anderson, W. F. & Anderson, C. M. *J. molec. Biol.* **104**, 197-222 (1976).
15. Stammers, D. K. & Muirhead, H. *J. molec. Biol.* **112**, 309-316 (1977).
16. Rossmann, M. G. & Argos, P. *J. molec. Biol.* **105**, 75-95 (1976).
17. Levitt, M. & Chothia, C. *Nature* **261**, 552-558 (1976).
18. Stammers, D. K. & Muirhead, H. *J. molec. Biol.* **95**, 213-225 (1975).
19. Rose, I. A. & Rieder, S. V. *J. biol. Chem.* **234**, 1007-1010 (1959).
20. Rose, I. A. *Brookhaven Symp. Biol.* **15**, 293-309 (1962).
21. Rose, I. A. *J. biol. Chem.* **235**, 1170-1177 (1960).
22. Schulz, G. E. & Schirmer, R. H. *Nature* **250**, 142-144 (1974).
23. Rossmann, M. G. & Argos, P. *J. biol. Chem.* **250**, 7525-7532 (1975).
24. Rao, S. T. & Rossmann, M. G. *J. molec. Biol.* **76**, 241-256 (1973).
25. Robinson, J. L. & Rose, I. A. *J. biol. Chem.* **247**, 1096-1105 (1972).
26. Phillips, D. C., Rivers, P. S., Sternberg, M. J. E., Thornton, J. M. & Wilson, I. A. *Biochem. Soc. Trans.* **5**, 642-647 (1977).

letters to nature

Dual character of the rapid burster and a classification of X-ray bursts

DIFFERENT kinds of X-ray bursts, as observed from the rapid burster (MXB1730-335) (refs 1, 2) and from other sources³⁻⁵, can be classified into two types which, as we will show, may have different origins and production mechanisms. Type I bursts occur at intervals of hours, days or longer. Their spectra almost always soften during burst decay, and their average spectrum during the first few seconds of a burst is generally harder than the spectrum of the associated persistent X-ray emission (if present). Type II bursts occur at intervals of several seconds to minutes, and their spectra do not soften during burst decay. The rapid burster¹⁻⁶ can produce up to several thousand type II bursts per day. Type II bursts may perhaps also be produced by other X-ray sources such as Cyg X-1 (ref. 7). Type I bursts are produced by all other burst sources reported so far³⁻⁵. Here we will show that the rapid burster also produces type I bursts.

Between 1977 September 27.27 and October 2.76 UT we observed the rapid burster (MXB1730-335) (ref. 1) with the y -axis detectors of the SAS-3 observatory⁸. The burst rate varied from $\sim 50 \text{ h}^{-1}$ to $\sim 250 \text{ h}^{-1}$, the burst durations from $\sim 3 \text{ s}$ to $\sim 10 \text{ s}$ (ref. 9).

In addition to the rapidly repetitive bursts (up to several thousand per day), henceforth called 'rapid' bursts, we detected 19 bursts which have a different appearance: (1) they

last longer ($\sim 30 \text{ s}$) than the rapid bursts; (2) they occur out of sequence of the rapid burst pattern, grossly violating the $E-\Delta t$ relations for rapid bursts^{1,4,6}; (3) their spectra soften during burst decay unlike the spectra of the rapid bursts; and (4) their time-averaged spectra during the first $\sim 10 \text{ s}$ are often harder than those of the rapid bursts.

Figure 1 shows, in addition to many rapid bursts, three of the 19 'special' bursts which were recorded in the horizontal tube system⁸ (1.3-12 keV). In several cases we detected a rapid burst coincident with a special burst (Fig. 1b and c), as would be expected (chance coincidence) if the two are unrelated. This may indicate separate origins and possibly different mechanisms for the two types of bursts.

Based on the similarity of the relative counting rates in the independent y -axis detectors for the special and rapid bursts, the 19 special bursts must have come from within $\sim 10 \text{ arc min}$ of MXB1730-335. We believe that they actually came from MXB1730-335. They did not come from MXB1728-34 (at $\sim 0.5^\circ$ distance)¹⁰ as we detected 11 bursts from this source with markedly different ratios of the counting rates in the y -axis detectors than the special bursts.

Figure 2 shows the data from five energy channels of the bursts shown in Fig. 1a. The softening of the spectrum during the decay of the special burst is clearly visible, but it is absent in the five rapid bursts. Also, unlike the rapid bursts, the special burst is very distinct in the 8-19 keV channel.

Table 1 lists the times when the 19 bursts were detected. The burst intervals between the detected bursts are plotted in Fig. 3.

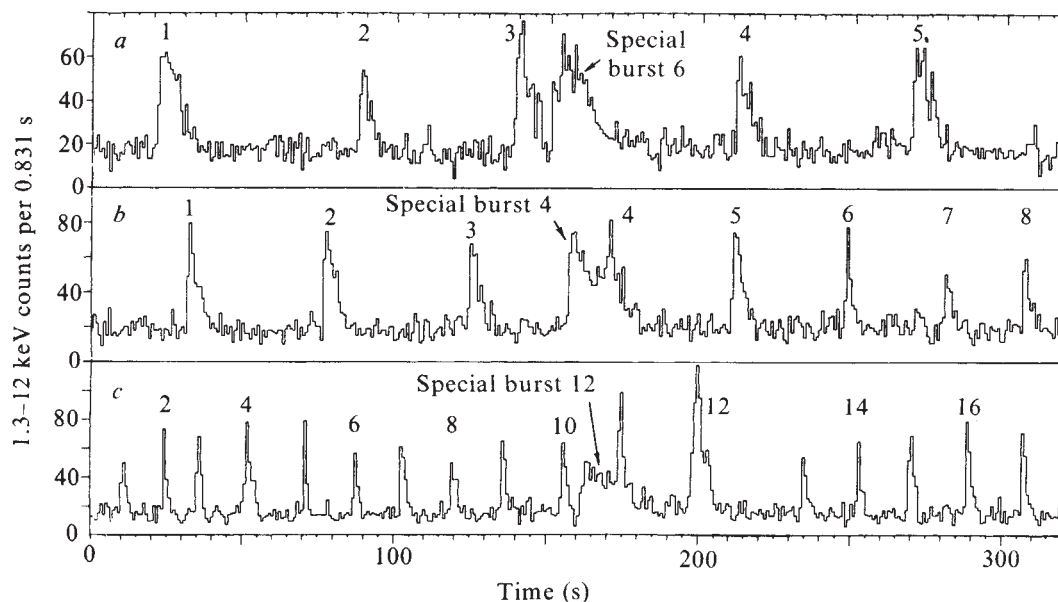


Fig. 1 Three sections of SAS-3 data from the horizontal tube detectors (sum of three channels, 1.3–12 keV). Each section contains a 'special' burst: *a*, burst 6; *b*, burst 4; *c*, burst 12 (see Table 1). The counting rates would have been somewhat higher if the rapid burster had been exactly in the centre of the field of view. Correction factors for the burst data are 1.11 (*a*), 1.05 (*b*) and 1.18 (*c*). The special bursts (type I) arrive out of sequence of the rapidly repetitive bursts (type II), which are independently numbered. They have approximately the same height as the 'rapid' bursts, but they last considerably longer (~ 30 – 40 s). In (*b*) and (*c*) a rapid burst occurs simultaneously with a special burst. Note in (*c*) that the special burst (12) is followed by a large rapid burst (no. 12) ~ 45 s later. Such a coincidence was observed four times (see text).

The minimum detected interval was 2.9h, the maximum was 20.4h. Some bursts were almost certainly missed due to Earth occultation.

There are no obvious regularities in the burst intervals (Fig. 3), but it is possible that a few semi-regular series were present with intervals near 3–4h (see dashed lines in Fig. 3). The regularity was clearly broken at times. We should point out that the burst intervals can, during certain periods, become synchronised (in phase) with the 30 min occulted portion of each 95 min orbit. For example, the mean burst intervals between bursts 8 and 13 (day 416–417 in Fig. 3) may have been ~ 3.1 h (r.m.s. jitter 15%), which is almost exactly twice the orbital period (1.57h). Therefore, the probability of missing four bursts in a row (near day 418; Fig. 3) is not necessarily low. Nevertheless, to fit all 19 bursts in one semi-regular series with intervals of ~ 3 –4h, spanning the entire 5.5d observing period, would require at least 18 bursts to have been blocked by the Earth; this probability is negligibly small.

Between September 30.0 and 30.8 (UT) 1977, the rapid burst rate was very high, ~ 160 – 220 bursts h^{-1} , compared to ~ 95 bursts h^{-1} averaged over the rest of the observation. During this period, the bursts were nearly equal in size, except that in every orbit, 1–4 larger rapid bursts were emitted (average ~ 2.6 h^{-1}) with 3–5 times more energy than the other bursts. Such large bursts were occasionally detected during the rest of the observation, but only in $\sim 10\%$ of the orbits. Of 45 such large rapid bursts detected in the entire 5.5 day observation, 25 were detected in the September 30.0 to 30.8 period. During this ~ 19 h period (~ 12.7 h of unocculted data on the rapid burster) we detected four special bursts (10–13 in Table 1), each of which was followed within ~ 80 s by such a large rapid burst (Fig. 1c). Given the observed rate of special bursts (~ 0.32 h^{-1}) and of large rapid bursts (~ 2.6 h^{-1}) during this interval, the expected number of such close coincidences is ~ 0.02 h^{-1} , or ~ 0.25 during the entire interval. The probability of observing four such close coincidences is thus $\sim 10^{-4}$. We therefore conclude that there is some causal connection between the special bursts and the rapid burst, and thus that the special bursts came from MXB1730–335.

The rapid bursts from the rapid burster are of type II, and are different from type I bursts in that they occur at a much higher

rate and do not show spectral softening during burst decay. The 19 special bursts are type I bursts. They resemble the bursts observed from many other sources^{3–5} in that: the time intervals between bursts (hours) are comparable; the burst recurrence patterns are similar (refs 5, 11, 12 and SAS-3 unpublished results); and the softening of the spectra during burst decay observed in the special bursts is characteristic of X-ray bursts from almost all sources^{3–5}.

The detection of type I bursts from the rapid burster may indicate that the rapid burster, in spite of its unique behaviour, is physically similar to other burst sources.

We have examined our March–April 1976 data from the rapid burster for the presence of special bursts. We found none in the March data but we found six bursts in the April data that are very similar to special bursts. They were in a semi-regular sequence with average burst intervals of ~ 3.8 h (r.m.s. jitter 10%), and the spectral softening during burst decay was very clear in at least two cases. The horizontal tube xenon detectors⁸ were turned off at the time; we therefore cannot tell whether the spectra of the six bursts were harder than that of the rapid bursts.

The rapid burst pattern was very similar in April 1976 and September–October 1977 (regular burst intervals) but very different in March 1976 (intervals differed by a factor of ~ 100) (refs 1–4). This may well be why special bursts were detected

Table 1 Times that special (type I) bursts were observed from MXB1730–335 between 1977 September 27.267 and October 2.760 (UT).

13.767 (Begin observation)	
1 13.9343	11 16.7352
2 14.2800	12 16.9575
3 14.7179	13 17.2161
4 14.8657	14 18.0655
5 14.9983	15 18.3693
6 15.1866	16 18.5970
7 15.7575	17 18.7156
8 16.0347	18 18.8616
9 16.3023	19 19.1000
10 16.6015	19.2596 (End observation)
Julian Day—2443400 days	

A type I burst occurring at 18.9854 UT was discovered in the data after this letter was submitted. This is not included in this table or in Fig. 3.

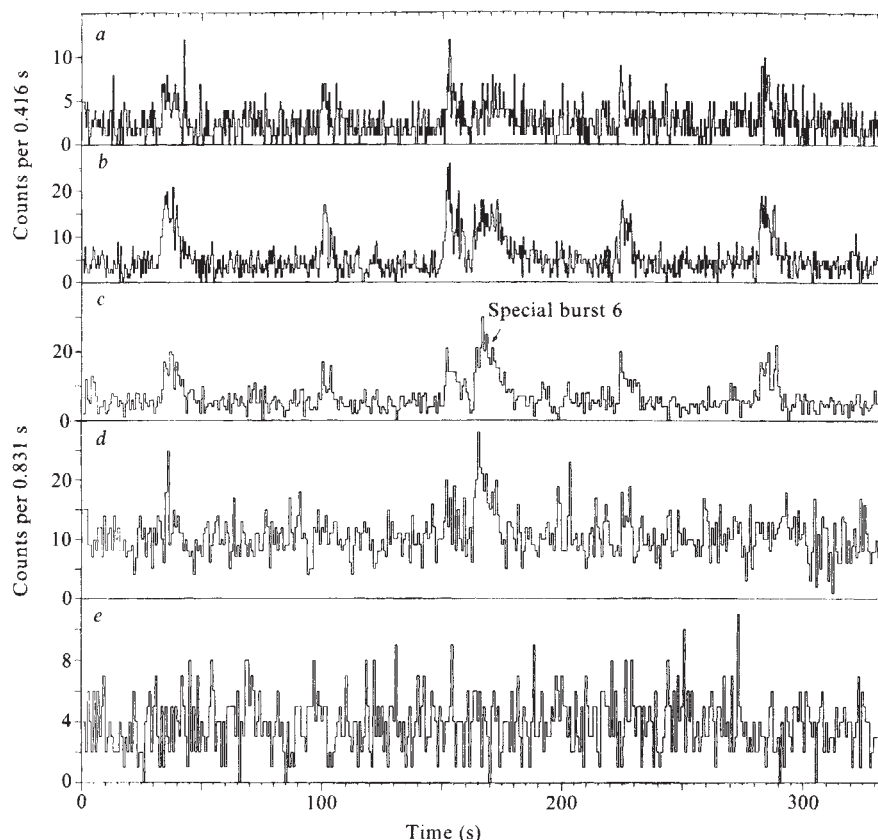
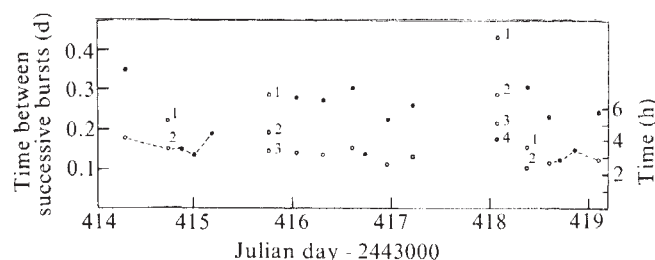


Fig. 2 Spectral information (five energy channels) for the data shown in Fig. 1a. *a*, 1.3–3 keV; *b*, 3–6 keV; *c*, 6–12 keV; *d*, 8–19 keV; *e*, 19–27 keV. Notice that the spectrum during burst decay of the special (type I) burst softens. This softening is very common in bursts from other sources, but it is absent in the rapid (type II) bursts. The special burst is clearly visible in the range 8–19 keV, and thus its mean spectrum (first ~ 10 s) is harder than that of the rapid bursts.

in April 1976 and September–October 1977 but not in March 1976. We did, however, detect 23 unusual bursts in March 1976 of which 17 were previously reported as ‘anomalous’ bursts by Ulmer *et al.*². They occurred at irregular intervals and all were preceded by a very energetic rapid burst. This established that the anomalous bursts came from the rapid burster and that there is a causal relation between the rapid and the anomalous bursts². The anomalous bursts have some features in common with the special bursts, and there are some differences. Both have durations of ~ 30 s, and both occur out of sequence of the rapid burst pattern, grossly violating the $E-\Delta t$ relations for rapid bursts^{3,4,6}. The ratio of the time-averaged flux of rapid bursts and special bursts was ~ 120 . This is similar to the ratio of ~ 140 for rapid bursts and anomalous bursts. The main differences between anomalous and special bursts are that anomalous bursts have lower peak luminosity compared to the most energetic rapid bursts, longer rise times, a softer mean spectrum,

Fig. 3 Intervals between detected bursts are shown as black dots. $\bigcirc$, Indicate the observed intervals divided by two, three, four and so on (bursts can be missed as a result of Earth blocking). The numbers next to the open circles indicate the number of possibly missing bursts. Semi-regular burst series may have occurred near day 415 (dashed line), between day 416 and 417, and near day 419 (dashed line) (see text).



and they probably lack the spectral softening during burst decay (although, because anomalous bursts are relatively weak, this latter point is not certain). We do not know whether anomalous and special bursts are related in that they are caused by the same phenomenon but we suspect that they are.

White *et al.*⁶ have discussed the previously well known breakdown of the linearity in the $E-\Delta t$ relation of rapid bursts (first reported by Lewin in December 1976) (ref. 4). They show four days of data of the rapid burster as observed in April 1977 with Ariel V. There are no special bursts in their data. Anomalous bursts might be present in the Ariel V data. But, the sensitivity of the Ariel V observations is probably too low to recognise them as the peak luminosity of anomalous bursts is $\sim 1/3$ that of rapid bursts² (but see note added in proof).

The rapid burster seems to have a dual character. On one hand, it can produce type I bursts similar to those observed from other burst sources. On the other hand, quite independently, it can produce type II bursts in rapid succession (up to $\sim 4,000$ per day). Bursts of type I and type II can occur simultaneously (Fig. 1b and c) which may indicate separate origins and possibly different mechanisms for their production. The two types of bursts can influence one another, however.

In order to find out whether there are times when the rapid burster produces only type I bursts, we examined several weeks of data from the nearby source MXB1728–34, during times that no rapidly repetitive bursts were observed from MXB1730–335 (refs 13–15 and SAS-3 unpublished results). We did not find any. This, of course, does not prove that it never happens, and we plan to observe the rapid burster frequently after the rapidly repetitive type II bursts have ceased. This is expected some time in November 1977, if the duration of rapid burster activity is the same as was observed in the springs of 1976 and 1977 (refs 1, 6, 15–17).

The ratio of the time-averaged luminosity of rapid bursts

(type II) to that of both 'special' bursts and 'anomalous' bursts (type I) was ~ 130 . This is comparable to the ratio of time-averaged persistent X-ray emission and time-averaged type I burst emission for many burst sources³⁻⁵. This opens up the interesting possibility (suggested by J. Doty, personal communication) that in MXB1730-335 the persistent X-ray emission as observed from many burst sources manifests itself in the form of rapidly repetitive type II bursts. In support of this, we point out that the mean burst spectra of rapid bursts are similar to the spectra of the persistent emission from most sources that produce bursts³⁻⁵. Also, the mean spectra (first ~ 10 s) of special (type I) bursts are harder than those of the rapid (type II) bursts just as the mean spectra of type I bursts from most sources are harder than that of the persistent emission³⁻⁵.

If this idea is correct, it may remove one of the main problems with thermonuclear flash models of X-ray bursts (ref. 5 and references therein), which predict the ratio of time-averaged steady luminosity to time-averaged burst luminosity to be $\lesssim 10^2$. No persistent X-ray emission has been detected from the rapid burster^{1,6}, and the ratio of steady luminosity to time-averaged type II burst luminosity has been measured to be as low as < 0.2 (ref. 6). But, if the rapidly repetitive type II bursts take the place of the steady emission and type I bursts have a thermonuclear origin, then the equivalent ratio would be ~ 130 for MXB1730-335.

Type II bursts as observed from the rapid burster are probably the result of instabilities in the accretion flow. The more common type I bursts may be the result of thermonuclear flashes or they too could result from some kind of an instability in the accretion flow (ref. 5 and references therein).

The brief flares as observed from the binary sources Cyg X-1 (ref. 7), GX304-1 (ref. 18) and LMCX-4 (ref. 19) meet the phenomenological definition of type II bursts. We do not discount the possibility that those flares are produced by the same mechanism that produces type II bursts in the rapid burster.

We thank Lynn Cominsky, John Doty and Claude Canizares for helpful discussions.

Note added in proof: We are informed by K. Mason (personal communication) that Ariel V would have detected anomalous bursts of the strength reported by Ulmer *et al.*² if they had occurred. Therefore, the rapid burster was producing neither special nor anomalous bursts in April 1977.

JEFFREY A. HOFFMAN
HERMAN L. MARSHALL
WALTER H. G. LEWIN

*Department of Physics and Center for Space Research,
Massachusetts Institute of Technology,
Cambridge, Massachusetts 02139*

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- Lewin, W. H. G. *et al. Astrophys. J. Lett.* **207**, L95 (1976)
- Ulmer, M. P., Lewin, W. H. G., Hoffman, J. A., Doty, J. & Marshall, H. *Astrophys. J. Lett.* **214**, L11 (1977).
- Lewin, W. H. G. *Mon. Not. R. astr. Soc.* **179**, 43 (1977).
- Lewin, W. H. G. *Ann. N.Y. Acad. Sci.* (in the press).
- Lewin, W. H. G. & Joss, P. C. *Nature* **270**, 211-216 (1977).
- White, N. E., Mason, K. O., Carpenter, G. F. & Skinner, G. K. *Mon. Not. R. astr. Soc.* (in the press).
- Canizares, C. & Oda, M. *Astrophys. J. Lett.* **214**, L119 (1977).
- Lewin, W. H. G. *et al. Mon. Not. R. astr. Soc.* **177**, 93P (1977).
- Hoffman, J. A., Marshall, H. & Lewin, W. H. G. *IAU Circ. No.* 3117 (1977).
- Hoffman, J. A. *et al. Astrophys. J. Lett.* **210**, L13 (1976).
- Li, F. K., Lewin, W. H. G., Clark, G. W., Doty, J., Hoffman, J. A. & Rappaport, S. A. *Mon. Not. R. astr. Soc.* **179**, 21P (1977).
- Lewin, W. H. G., Hoffman, J. A., Doty, J., Li, F. K. & McClintock, J. E. *IAU Circ. No.* 3075 (1977).
- Hoffman, J. A., Lewin, W. H. G. & Doty, J. *Mon. Not. R. astr. Soc.* **179**, 57P (1977).
- Lewin, W. H. G., Doty, J., Hoffman, J. A. & Li, F. K. *IAU Circ. No.* 2984 (1976).
- Lewin, W. H. G. & Hoffman, J. A. *IAU Circ. No.* 3079 (1977).
- Hoffman, J. A. *IAU Circ. No.* 2953 (1976).
- Lewin, W. H. G. *IAU Circ. No.* 3067 (1977).
- McClintock, J. E., Rappaport, S. A., Nugent, J. & Li, F. K. *Astrophys. J. Lett.* **216**, L15 (1977).
- Epsstein, A. *et al. Astrophys. J.* **216**, 103 (1977).

The nature of Aquila X-1

THE unusual X-ray source Aquila X-1 (=4U1908+00; =2S1908+005) has been observed for almost a decade, and represents a singular link between the 'steady' galactic X-ray stars and the flaring and transient sources. Although this object normally has an X-ray flux of the order 1-10% of the Crab Nebula¹⁻³, approximately once per year it undergoes an intense outburst to a level comparable to the Crab²⁻⁴. A 1.3-d X-ray periodicity during the outburst has also been suggested⁵. A recent very accurate X-ray position for Aql X-1⁶ has permitted the optical identification of the source⁷ using primarily the extensive plate material described by Davidsen *et al.*². We report here photometry and spectroscopy of the counterpart and the stars in the immediately surrounding field, and use these data to arrive at some inferences regarding the nature of this unusual X-ray system.

Photoelectric photometry of nine stars in a 1 arc min field about Aql X-1 was obtained in July and August 1976, using the 2.1 m reflector of the Kitt Peak National Observatory with the Cassegrain computer-photometer. Standardisation of these *UBV* data was achieved through observations of standard stars from the compilation of Landolt⁸; the objects observed were in the range $12.4 < V < 17.1$, $13.1 < B < 18.3$. Spectroscopy of nine stars in the field, partially overlapping with the photometric sample and ranging $12.4 < V < 15.2$, was obtained in November 1975 using the Mk I multichannel spectrometer⁹ at the 1.3 m reflector of the McGraw Hill Observatory. Spectral types were derived for these stars through comparison with spectral standards¹⁰ and through H α equivalent widths¹¹.

We have also obtained photographic photometry of the optical counterpart in quiescence and outburst. The quiescent data consist of direct photographic plates obtained with the 0.9 m Crossley reflector of the Lick Observatory in August and October 1977. These plates were calibrated through iris photometry of the stars in the photoelectric sequence described above, providing a direct comparison sequence extending to $V=17$, $B=18$, and through a transfer from the M15 field described by Sandage¹², which is photoelectrically calibrated to $V=22$, $B=23$. This combined photoelectric-photographic calibration of the Aql X-1 field is considerably more accurate than a standard photographic transfer, judging from the scatter in the magnitudes derived for the standard stars, which is of the order 0.10-0.15 mag even for the faintest objects. The outburst data consist of the Palomar Observatory Sky Survey prints which remarkably show Aql X-1 near maximum light on the date (August 23-24 1951) they were exposed. The *B* and *R* magnitudes of Aql X-1 on these prints were determined by measuring on a 2-axis Mann measuring engine image diameters of the comparison stars discussed above.

We find that for the Aql X-1 counterpart in quiescence, $V=19.4 \pm 0.15$, and $(B-V) \gtrsim 1.0$; a *B* image is only barely detectable on our plate material. During outburst we find $B=18.41 \pm 0.04$, $R=17.37 \pm 0.24$. Using a colour equation applicable to the Sky Survey¹³, we infer $(B-V)=0.7 \pm 0.2$. An uncalibrated outburst plate described by Thorstensen¹⁴ indicates that the full range of variability may in fact be slightly larger, as in the *B* band the counterpart reached a brightness comparable to star no. 3 in the chart of ref. 6. For that star our photoelectric data yield $B=17.8$. Assuming a reasonably constant colour near the outburst peak, we adopt $V=17.1$, $(B-V)=0.7$ for the outburst state. The large range of inferred photometric variability, together with the simultaneity of two X-ray and optical outbursts⁷ makes the identification of the optical counterpart (star 5 in ref. 6) secure.

Our photometry and spectroscopy of the stars in the immediately surrounding field yield information on the interstellar extinction in this direction. For the stars with spectroscopy, we used our photoelectric or photographic magnitudes and colours to estimate colour excesses and distances; six of

these objects prove to be foreground K stars and thus not distant enough to provide a useful probe. For three additional stars with photoelectric *UBV* data but no spectra, the 'Q' method¹⁵ indicates *B* spectral types, again permitting extinction and distance estimates. The six stars for which we have useful data span a range in distance of 0.3–5 kpc and in A_V of 1.5–4.4 mag. We find these data to be consistent with an exponential model¹⁶ for the distribution of dust perpendicular to the galactic plane, and with the parameters given by Allen¹⁷ of $A_V/d=1.9$ mag kpc⁻¹ in the plane and scale height $H=130$ – 190 pc. The line of sight to Aql X-1 breaks out of the dust layer at a distance of 1.9–2.7 kpc and has a limiting visual extinction A_V of 4–5 mag in this model.

Using this model for the interstellar extinction, we have transformed our observed brightness and colour of Aql X-1 during quiescence and outburst to luminosity and intrinsic colour as a function of assumed distance. The spectral observations¹⁴ suggest a quiescent spectral class near K0. Our quiescent brightness data place dwarf stars of this approximate spectral class at distances between 1 and 2 kpc. Giant stars of the observed spectral class are ruled out by the colours and our reddening against distance relation. In particular, a K0 dwarf at a distance of 1.6 kpc is in good agreement with all the data, and implies an outburst X-ray luminosity far below the Eddington limit.

The existing data, though fragmentary, have interesting implications. The absence of emission lines in the quiescent spectrum¹⁴, despite the detection of an absorption feature, emphasises the difference between Aql X-1 and the dwarf novae, which have emission spectra at all times. This is in contrast to the suggestion⁴ that Aql X-1 may be similar to the dwarf novae. Most striking, however, is the high ratio of X-ray to optical intensity. At maximum light, using our *V* magnitudes and the observed X-ray fluxes of Kaluziński *et al.*⁴, we find for this ratio a value of about 5,000; in the quiescent state, the ratio is lower but still at least 500. These values should be compared to other identified X-ray sources; the largest value is probably that of Sco X-1, which has a ratio of about 1,000 (see ref. 18). Aql X-1 is even more outstanding than this extra factor of 5 indicates, because in Sco X-1 the visible light source has a temperature $\sim 30,000$ K and a bolometric correction of several magnitudes, probably reducing the bolometric X-ray to optical ratio to $\sim 10^3$. Our colour measured for Aql X-1, together with the indication¹⁴ of a late G or early K type spectrum when quiescent, suggests a reddening $E(B-V)$ of a few tenths of a magnitude and a small bolometric correction during outburst. Correction for extinction decreases the outburst X-ray to optical ratio, but probably not below 500.

The fact that the quiescent spectrum¹⁴ is a late-type absorption spectrum implies that most of the observed optical luminosity is generated within the star. The photospheric opacity of a K dwarf, for example, is about $0.1 \text{ cm}^2 \text{ g}^{-1}$, less than the opacity of matter to any X-rays. Therefore X-ray heating sufficient to substantially affect the atmospheric heat balance during quiescence would produce a strong chromosphere and an emission spectrum, as in Sco X-1^{18,19}; the absence of these implies that the atmosphere is heated from within the star. This is consistent with the fact that the X-ray brightness increases an order of magnitude more than the visible brightness from quiescence to flare.

An X-ray to optical luminosity ratio of 500 implies, in the conventional semidetached Roche binary model, that the companion's radius is about 0.1 of the separation²⁰; equivalently, the X-ray source is about 100 times more massive than its companion. This conclusion is independent of the distance, except through the extinction, but requires that the X-ray radiation should not be highly anisotropic. Three possible solutions to the problem posed by the small size of the companion are a companion of less than stellar mass, a super-massive X-ray source, or abandonment of the assumption that the system is semidetached. The first is ruled out by the fact that in quiescence the companion's luminosity comes

from within, and the second is possible, but will be thought speculative by most. The third explanation, which we favour, poses the problem of how mass transfer is brought about. Oke²¹ argues that the X-ray transient A0620–00 (=V616 Mon; =Nova Mon 1975) is also a K dwarf and presents a similar problem.

If our suggested model for Aql X-1 is correct, it should show (for favourable inclination) a strong photometric periodicity during outburst, perhaps resembling HZ Her, but not when quiescent. During outburst, there may also be an emission spectrum, as in Sco X-1 and A0620–00. There could be a detectable spectroscopic orbit. With our estimated binary separation, a total system mass of $2M_\odot$ and a dwarf companion, an orbital period of about 2 d is implied, possibly consistent with the 1.3-d X-ray period reported by Watson⁵.

Thus, perhaps the most important implication of the data is that Aql X-1 may be the second known case of a low mass galactic X-ray source where the semi-detached Roche model, previously widely used for these systems, is not applicable. We also have strong evidence that the primary is a dwarf and that X-ray heating is the source of the optical outbursts.

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BRUCE MARGON
JONATHAN I. KATZ

Department of Astronomy and Institute of
Geophysics and Planetary Physics,
University of California,
Los Angeles, California 90024

LARRY D. PETRO

Hale Observatories,
Pasadena, California 91101

Received 28 October; accepted 17 November 1977.

1. Friedman, H., Byram, E. T. & Chubb, T. A. *Science* **156**, 374–376 (1967).
2. Davidsen, A., Sanford, P. W., Davison, P. & Mason, K. *IAU Circ.* No. 2793 (1975).
3. Buff, J. *et al. Astrophys. J.* **212**, 768–773 (1977).
4. Kaluziński, L. J., Holt, S. S., Boldt, E. A. & Serlemitsos, P. J. *Nature* **265**, 606–607 (1977).
5. Watson, M. G. *Mon. Not. R. astr. Soc.* **176**, 19p–23p (1976).
6. Doxsey, R. E. *et al. Nature* **269**, 112–116 (1977).
7. Thorstensen, J. R. *et al. IAU Circ.* No. 3088 (1977).
8. Landolt, A. U. *Astr. J.* **78**, 959–981 (1973).
9. Shectman, S. A. & Hiltner, W. A. *Publs astr. Soc. Pacif.* **88**, 960–965 (1976).
10. Abt, H. A., Meinel, A. B., Morgan, W. W. & Tapscott, J. W. *An Atlas of Low Dispersion Grating Stellar Spectra* (1968).
11. Pritchett, C. & van den Bergh, S. *Astrophys. J. Suppl.* **34**, 101–114 (1977).
12. Sandage, A. *Astrophys. J.* **162**, 841–870 (1970).
13. King, I. R. & Raff, M. I. *Publs astr. Soc. Pacif.* **89**, 120–121 (1977).
14. Thorstensen, J. R. paper presented at *First Pacific X-ray Astrophysics Colloquium*, Stanford, California (1977); *Astrophys. J. Lett.* (in the press).
15. Johnson, H. L. & Morgan, W. W. *Astrophys. J.* **117**, 313–352 (1953).
16. Mihalas, D. M. & Routly, P. M. *Galactic Astronomy* (W. H. Freeman, San Francisco, 1968).
17. Allen, C. W. *Astrophysical Quantities* 3rd edn. (Athlone, London, 1973).
18. Katz, J. I. *Ann. N.Y. Acad. Sci.* **262**, 400–403 (1975).
19. Katz, J. I. *Astr. Astrophys.* **39**, 241–244 (1975).
20. Kuiper, G. P. & Johnson, J. R. *Astrophys. J.* **123**, 90–94 (1956).
21. Oke, J. B. *Astrophys. J.* **217**, 181–185 (1977).

γ -Rays from γ Geminorum?

THE γ -ray source CG195+4 (Geminga) discovered by SAS 2 and further observed by COS B has a period of 59 s which is increasing at a rate $\dot{P} = 2 \times 10^{-9}$ (refs 1,2). If the periodicity is produced by a neutron star which is spinning down at the observed rate, then the rate at which rotational energy is being lost is $L \sim 3 \times 10^{32} \text{ erg s}^{-1}$ (ref. 3). The observed γ -ray flux of $3 \times 10^{-6} \text{ photon cm}^{-2} \text{ s}^{-1}$ with energy $E > 100 \text{ MeV}$ implies that the distance, d , to the source is given by $d \approx 73^{1/2} \text{ pc}$ where $f(\approx 1)$ is the fraction of the total energy output which is emitted as γ -rays. Theoretical studies

of the evolution of binary systems which give rise to the binary X-ray sources have led to the suggestion that, before the neutron star starts to accrete and spin up (that is, before the X-ray source turns on) it spends a comparatively long time being spun down in the weak stellar wind of its companion⁴. This leads us to seek a candidate companion for the γ -ray source which is an early type star at a distance of $\gtrsim 70$ pc. As we report here, the only object which satisfies these constraints is γ Geminorum.

The star γ Gem has $m_v = 1.9$ mag and has been classified as A0IV (ref. 5) and A1III (ref. 6). Parallax measurements yield a distance estimate of $d \sim 30$ pc (ref. 7). We therefore require $f \sim 0.17$. We assume the star to have a bolometric luminosity $L_{\text{bol}} \sim 6 \times 10^{35}$ erg s⁻¹, a mass $M_1 \sim 3M_{\odot}$ and a radius $R_1 \sim 3.5 \times 10^{11}$ cm. If we consider a neutron star orbiting γ Gem at a distance comparable to R_1 then a substantial fraction ($\sim 1/4$) of the primary's stellar wind will be accreted towards the companion at a rate $\dot{M}_A$.

We assume that the neutron star is strongly magnetic and that its corotation radius ($R_{\Omega} \sim 2.3 \times 10^9$ cm) is roughly equal to the Alfvén radius of its magnetosphere, R_M . The neutron star is in its 'sleeping phase': that is, material is not accreted down to the stellar surface but is, rather, expelled from the vicinity of the Alfvén surface. The energy to do this comes from the rotational energy of the neutron star, and if the material is thrown off at about the escape velocity from the region of the Alfvén radius, $V_M \simeq (2GM/R_M)^{1/2}$, we may write $L \sim G\dot{M}_A/R_M$. This implies that $\dot{M}_A \simeq 5 \times 10^{15}$ g s⁻¹, so that $\dot{M}_W \sim 2 \times 10^{16}$ g s⁻¹. Considering that mass loss will be enhanced by the presence of a close companion this is a reasonable value for the mass loss rate of a star like γ Gem (ref. 8). Making use of the definition of the Alfvén radius, $B^2(R_M)/8\pi \sim 1/2 \rho_M V_M^2$ where $\rho_M = \dot{M}_A/(4\pi R_M^2 V_M)$ is the density of the gas at R_M , we obtain the surface field (assumed dipolar) of the neutron star to be $B \approx 7 \times 10^{12}$ gauss. We have taken the neutron star to have mass $1 M_{\odot}$ and radius 10^6 cm.

Despite the assumed equality of R_M and R_{Ω} material does not accrete down to the stellar surface because at the low density and the high temperature to which the matter is heated ($T \sim 1/2 m_{\text{p}} v_M^2/k \sim 7 \times 10^8$ K) it cannot cool sufficiently by bremsstrahlung to accrete^{9,10}. The energy loss rate due to bremsstrahlung radiation is about 10^{30} erg s⁻¹ which is much less than L , the rate of energy generation at the magnetosphere. This energy must take the form of bulk motion of the gas and hence the gas is expelled.

To produce the γ -rays we suggest that a fraction of the energy released in the interaction between the gas and magnetic field at R_M is in the form of relativistic electrons with $\gamma \sim 2 \times 10^3$. If about a half of these penetrate the magnetosphere, where they radiate by synchrotron in the extreme ultraviolet (~ 400 Å), the dominant loss process for the remainder is inverse Compton scattering on these ultraviolet photons, resulting in the production of the observed 100 MeV γ -rays. The electron energy-loss timescale for this process, t_{IC} , is less than half the pulse period if the electrons are accelerated only over a fraction $\gtrsim 1/4$ of the magnetosphere. This seems likely, given a non-axisymmetric magnetosphere and, together with asymmetries in the initial velocity distribution of the electrons producing the γ -rays, could give rise to a variation in the γ -ray flux with a period equal to (or half) the rotation period of the neutron star. We may expect the ultraviolet synchrotron radiation to have a luminosity and periodic variation similar to those of the γ -rays. The timescale for γ -ray production by scattering of stellar photons, $t'_{\text{IC}} \sim 10^3$ s $\gg t_{\text{IC}}$.

Taking the binary separation $a \sim 2R_1 \sim 7 \times 10^{11}$ cm, a rough estimate of the orbital period is $P_{\text{orb}} \sim 1.5$ d. Our estimate of the radial velocity of γ Gem would be $\sim 70 \sin i$ km s⁻¹, where i is the orbital inclination. To be consistent with radial velocity observations^{6,7,11}, we may require i to be small. This would also tend to make detection of the doppler shift of the γ -ray pulse difficult. We note that when γ Gem has evolved to fill its Roche lobe and starts to transfer material at a higher rate, the neutron star will turn on as an X-ray source with properties very similar to those of Her X-1 (which consists of a neutron star orbiting an F subgiant with an orbital period of 1.7 d).

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R. E. DAVIES
A. C. FABIAN
J. E. PRINGLE

*Institute of Astronomy,
Madingley Road,
Cambridge, UK*

Received 10 November; accepted 30 December 1977.

1. Thompson, D. J. *et al. Astrophys. J.* **213**, 252–262 (1977).
2. Masnou, J. L. *et al. Proc. 12th ESLAB Symp.* (1977).
3. Maraschi, L. & Treves, A. *Astr. Astrophys.* **61**, L11–L13 (1977).
4. van den Heuvel, E. P. J. *Proc. 8th Texas Symp., New York Acad. Sci.* (in the press).
5. Johnson, H. L. & Morgan, W. W. *Astrophys. J.* **117**, 313–352 (1953).
6. Palmer, D. R., Walker, E. N., Jones, D. H. P. & Wallis, R. E. *R. Obs. Bull.* No. 135 (1968).
7. Beardsley, W. R. *J.A.U. Symp.* No. 30 (eds Batten, A. H. & Heard, J. F.) (1967).
8. Reimers, D. *Proc. I.A.U. Coll.* No. 42, Bamberg, (in the press).
9. Arons, J. & Lea, S. M. *Astrophys. J.* **207**, 914–936 (1976).
10. Elsner, R. F. & Lamb, F. K. *Astrophys. J.* **215**, 897–913 (1977).
11. Harper, W. E. *Publ. Dom. astr. Obs.* **6**, 223 (1935).

Evidence that cosmic γ -ray bursts are galactic

OBSERVATIONS from our latest balloon flight and a burst observed by Nishimura *et al.* combined with our previous results and the observations of Bewick *et al.* provide strong evidence that cosmic γ -ray bursts are galactic. It also seems that the recently discovered X-ray bursts and γ -ray bursts have a common origin. A further 41 h of balloon flight time with the University of California, Riverside (UCR) double Compton scatter γ -ray telescope gave no additional γ -ray bursts. This flight time is added to the earlier 24 h observation of Herzo *et al.*¹ who found three bursts with total energy greater than 1×10^{-6} erg cm⁻² and one burst with total energy greater than 4×10^{-6} erg cm⁻² to give lower values for the number of bursts of

$$\left(8 \begin{smallmatrix} +8 \\ -3 \end{smallmatrix}\right) \times 10^2 \text{ and } \left(3 \begin{smallmatrix} +7 \\ -1 \end{smallmatrix}\right) \times 10^2 \text{ d}^{-1}, \text{ respectively.}$$

Our new observations were carried out on a flight launched from Muskogee, Oklahoma on 24 May 1976 at 0100 UT. The altitude of the observations varied from 3.1 to 7.5 g cm⁻² of residual atmosphere. The γ -ray telescope is described elsewhere^{2,3}. The γ rays were detected in three different modes: first, total neutral counts in the large upper liquid scintillator tank (S1) that is $1 \text{ m} \times 1 \text{ m} \times 12.5$ cm; and second, total neutral coincidences between S1 and the lower scintillator tank (S2), $1 \text{ m} \times 1 \text{ m} \times 20$ cm. Third, γ rays whose energy and angle were identified by scatters in one of the 28 cells in S1 followed by a scatter in one of the 28 cells in S2. For each of these events the cell identification, energy deposit in S1 and S2 and the time of flight from S1 to S2 were recorded. S1 and S2 were each completely surrounded by plastic scintillator and charged particle events were vetoed with an efficiency of better than 99.99%.

The new UCR cosmic γ -ray burst frequencies are given in Fig. 1 along with other satellite and balloon values. The satellites, Vela, IMP 7 and SAS II report observations mainly for larger bursts of 10^{-5} to 10^{-4} erg cm⁻² because of the small sizes and sensitivities of their detectors. The reported Vela burst frequencies^{4,5} bend over at the lower burst sizes presumably because their trigger mechanism is size dependent. Their burst detection frequency then drops rapidly for the smaller bursts. But, the balloon observations with large areas and high sensitivities are able to observe burst sizes as low as $\sim 10^{-7}$ erg cm⁻². Some balloon observations, limited to times of less than about 24 h, have given upper limits only^{6–8}. The 1σ confidence

upper limit of Carter *et al.*⁸, shown in Fig. 1, falls much lower than the other 2σ upper limits. The 2σ confidence upper limit would be higher than the plotted value by a factor of 3. Cline and Schmidt⁹ argue that it should be plotted at a different position (solid rectangle). However, Carter *et al.*¹⁰ insist that their original position is correct. γ -Ray bursts with energies greater than about 10^{-6} and 10^{-7} erg cm⁻² have been observed over a few days and a few hours, respectively. Bewick *et al.*¹¹ pointed out that their point is compatible with a galactic source distribution. Nishimura *et al.*¹² published information about their burst but its contribution to the burst size distribution is reported here for the first time.

If the sources of the bursts are isotropic, either very close, less than a few hundred pc away, or very far—extragalactic—the burst distributions in size (S) should vary as $S^{-3/2}$. On the other hand, if the bursts are galactic and limited to a disk, they should vary as S^{-1} .

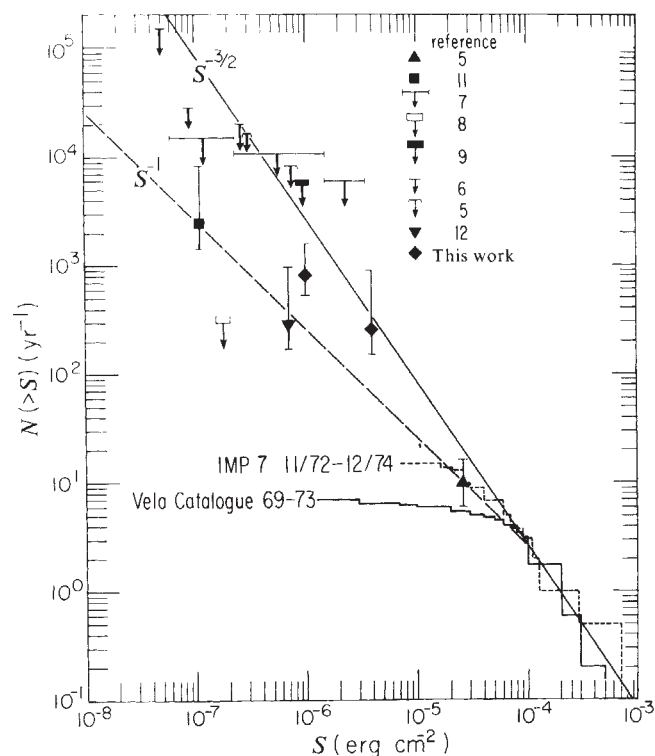


Fig. 1 γ -ray burst frequency distribution. Our points, those of Bewick *et al.*¹¹ and Nishimura *et al.*¹² have error bars of one standard deviation based on a Poisson distribution. The upper limit values are all two standard deviations (95% confidence level) of Gaussian distributions of the backgrounds except for the point of Carter *et al.*⁸ which is 1σ .

The five bursts observed by detectors on balloons along with the observations from satellites at larger burst sizes strongly suggest that the γ -ray bursts are galactic. The larger bursts, seen by satellites, are probably near, within a few hundred pc, and the smaller ones seen by balloons are probably much further away, perhaps thousands of pc.

It is of considerable astrophysical interest to determine whether the recently discovered X-ray bursts¹³ and the γ -ray bursts have a common origin. Their rise times of a fraction of a second and durations of a few seconds seem similar. Although a recent attempt to measure coincidences between the two types of events on the same satellite gave inconclusive results¹⁴, it seems that the evidence supports the same source for the two types of bursts. The γ -ray bursts are then the high energy tails of the low energy X-ray bursts.

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R. S. WHITE
J. M. RYAN
R. B. WILSON
A. D. ZYCH

Physics Department and
Institute of Geophysics and Planetary Physics,
University of California, Riverside,
Riverside, California 92521

W. B. DAYTON

Physics Department,
California State University,
Los Angeles, California 90032

Received 20 September; accepted 1 December 1977.

- Herzo, D., Dayton, B., Zych, A. D. & White, R. S. *Astrophys. J. Lett.* **203**, L115-L118 (1976).
- Herzo, D. *et al.* *Nucl. Instr. Meth.* **123**, 583-597 (1975).
- Zych, A. D. *et al.* *IEEE Trans. Nucl. Sci.* **NS-22**, 605-610 (1975).
- Strong, I. B. & Klebesadel, R. W. *Nature* **251**, 396-397 (1974).
- Cline, T. L., Desai, U. D., Schmidt, W. K. H. & Teegarden, B. J. *Nature* **266**, 694-696 (1977).
- Cline, T. L. & Desai, U. D. *Astrophys. J. Lett.* **196**, L43-L46 (1975).
- Johnson, W. N., Kurfess, J. D. & Bleach, R. D. *Astrophys. Space Sci.* **42**, 35-42 (1976).
- Carter, J., Dean, A. J., Manchada, R. K. & Ramsden, D. *Nature* **262**, 370-371 (1976).
- Cline, T. L. & Schmidt, W. K. H. *Nature* **266**, 749-750 (1977).
- Carter, J. C., Dean, A. J., Manchada, R. K. & Ramsden, D. *Nature* **266**, 750 (1977).
- Bewick, A., Coe, M. J., Mills, J. S. & Quenby, J. J. *Nature* **258**, 686-687 (1975).
- Nishimura, J. *et al.* *Proc. 15th Int. Conf. Cosmic Rays*, Plovdiv, **1**, 179 (1977).
- Lewin, W. H. G. *et al.* *Nature* **267**, 28-30 (1977).
- Quenby, J. J., Coe, M. J. & Engel, A. R. *Proc. 15th Int. Conf. Cosmic Rays*, Plovdiv **1**, 157 (1977).

Statistical investigation of compact emitting regions in extragalactic radio sources

EXTRAGALACTIC radio sources of high total luminosity usually contain compact emitting regions. These may be coincident with the optical galaxy or quasar, or they may be situated at the outer edges of extended double structures. Radio interferometers sensitive to structures with an angular scale ≤ 1 arc have been used at Jodrell Bank to investigate the frequency of occurrence of such compact regions. Large samples of sources, covering a wide range of flux densities, were studied. This work extends to lower flux densities than previous investigations based on observations of sources showing interplanetary scintillations^{1,2}. The frequency of occurrence of compact regions varies as a function of flux density. This is best explained as a cosmological effect involving the strong evolution of source properties with redshift. Several forms of this evolution, similar to those deduced from the radio source counts (see ref. 3), and the relationship between largest angular size and flux density^{4,5} are discussed elsewhere⁶.

This study is based on observations made with the Jodrell Bank-Defford interferometer (127 km baseline) at 408 MHz using two techniques. The instrument has been used as a drift interferometer to observe sources from the Bologna Catalogue^{7,8} in the declination range $29^\circ 30'-33^\circ$. A total of 713 sources were observed near meridian transit for approximately 3 min each as they moved through the aerial beams. The fringe visibility (γ_T) in position angle $\simeq 180^\circ$ was thereby determined for each source. Although γ_T for any source will depend on its detailed structure, the fraction $p(\gamma_T)$ of sources which have $\gamma_T > 0.5$ indicates the frequency of occurrence of compact regions (≤ 1 arc s) emitting over 50% of the flux density. The results of this survey have been examined as a function of flux density by dividing the sources into five samples which have median values of flux density ranging from 2.95 Jy to 0.45 Jy

Table 1 Drift observations of 713 radio sources from the B2 catalogue^{7,8} at 408 MHz

Median flux density (Jy)	Range of flux density (Jy)	No. of sources	Percentage with $\gamma_T \geq 0.5$
2.95	$2 \leq S < 5$	46	17.4 ± 5.6
1.2	$1 \leq S < 2$	126	19.0 ± 3.5
0.83	$0.7 \leq S < 1$	118	24.0 ± 5.0
0.59	$0.5 \leq S < 0.7$	247	19.4 ± 2.5
0.45	$0.4 \leq S < 0.5$	176	21.0 ± 3.1

as indicated in Table 1; errors of one standard deviation arising from counting errors (and from noise in the two lowest ranges of flux density) are shown. Within the errors, $p(\gamma_T)$ is constant at $\sim 20\%$ over this range at flux density.

Results have been obtained over a much wider flux density range by making tracking observations with the same interferometer. Studies have been made of 385 sources selected without bias from catalogues compiled at 408 MHz. Most of the sources were observed for a 9–12 h period within the hour angle range 1400–0900 h. During such a period the resolving power of this interferometer varies by less than a factor of two, but its position angle changes by more than 120° . The maximum fringe visibility (γ_m) from such observations then gives a better value for the fraction of the flux density coming from regions ≤ 1 arc s as it is largely independent of the effects of any double structure in the source or of elongation of the compact components.

These 385 sources have been divided into nine samples with median values of flux density ranging from 29 Jy to 0.047 Jy as indicated in Table 2. Far more information is available about the structures of intense sources than those of the fainter ones, but in order to search for any trend of compactness with flux density it is desirable to use a statistic which can be extracted, with equal reliability and objectivity, for all the sources being studied. The quantity $p(\gamma_m)$ (fraction of sample with $\gamma_m > 0.5$) is suitable and is plotted in Fig. 1. The values of $p(\gamma_m)$ increase from $11.5\% \pm 8\%$ for the most intense sources to $39\% \pm 10\%$ at 1.6 Jy, but then at lower fluxes remain constant at $\sim 30\%$ or possibly show a slow decrease. For the range of flux densities where the results for the drift and tracking

from 0.12 at 20 Jy (408 MHz) to 0.4 at 2.7 Jy (408 MHz) (the flux densities being converted to 408 MHz using a spectral index of -0.7). These results are clearly consistent with our observations above 1 Jy.

A detailed discussion of the present results will be published elsewhere⁶. Using a standard cosmological model and available data on a sample of sources from the 3CR catalogue as a basis for calculation, it is found that the observed relation, an increase in $p(\gamma_m)$ down to 1 Jy, and a constant value or decrease at lower fluxes, is difficult to explain unless there is a strong evolution of source properties with red shift. The calculations give the best agreement with the observations when a strong evolution of source density³ is coupled with a linear size evolution⁶ of the type $l \propto l_0 (1+z)^{-1}$. Linear size evolution has previously been used to explain the observed form of the largest angular size/red shift relation for QSOs¹⁷ and the largest angular size/flux density relation for radio sources in general⁵. This work suggests that it occurs within the compact emitting regions as well.

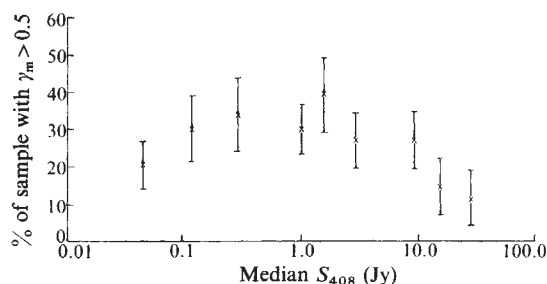


Fig. 1 The fraction of radio sources observed to emit more than half their flux from regions smaller than 1 arc s, as a function of flux density. Errors of 1σ are shown; these are determined from the numbers of sources in the samples and the distribution of individual values of γ_m within each sample.

Further interferometric observations of weak radio sources are in progress in an attempt to reduce the errors in Fig. 1 for the points below 1 Jy.

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B. ANDERSON
H. P. PALMER
*P. J. RICHARDS
B. SPEED
†R. S. WARWICK

University of Manchester,
Nuffield Radio Astronomy Laboratories,
Jodrell Bank, Macclesfield, Cheshire, UK

Table 2 Tracking observations of 385 radio sources at 408 MHz

Median flux density (Jy)	Range of flux density (Jy)	No. of sources	% with $\gamma_m \leq 0.5$	Catalogues and observers of compact emitting regions
29	> 20	17	11.5 ± 8	3CR ⁹ , Bridle ¹⁰ , obs. ref.
15	$12.2 < S < 20$	20	15 ± 8.5	
9.5	$8.0 < S < 12$	43	27 ± 8	
3.0	$2.5 < S < 4.5$	45	27 ± 7.5	B2 ^{11,8} , obs. ref. 12
1.6	$1.3 < S < 2.5$	33	39 ± 10	
1.0	$0.90 < S < 1.3$	58	33 ± 7	B2 ^{7,8} , obs. ref. 13
0.30	$0.20 < S < 0.90$	42	34 ± 10	
0.12	$0.090 < S < 0.20$	38	30 ± 9	5C2 ¹⁴ , 5C3 ¹⁵ , obs. ref. 16
0.047	$0.023 < S < 0.09$	89	21 ± 6.5	

observations can be compared, the values of p are consistent. It is found that $p(\gamma_m) \simeq 1.64 p(\gamma_T)$, showing that, for these compact regions, the effects of double structure and component elongation are appreciable but not overwhelming.

Swarup and Bhandari² have investigated the average compactness of radio source components as a function of flux density, using observations of interplanetary scintillation at 81.5, 327 and 430 MHz. They found that the median value of scintillation visibility (a parameter comparable to γ_m) increased

Present addresses: *The Appleton Laboratory, Ditton Park, Slough, Berkshire, UK.
†University of Leicester, Department of Physics, University Road, Leicester, UK.

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1. Readhead, A. C. S. & Longair, M. S. *Mon. Not. R. astr. Soc.* **170**, 393–404 (1975).
2. Swarup, G. & Bhandari, S. M. *Astrophys. Lett.* **17**, 31–36 (1976).
3. Doroshkevich, A. G., Longair, M. S. & Zeldovich, Ya. B. *Mon. Not. R. astr. Soc.* **172**, 501–512 (1975).
4. Swarup, G. *Mon. Not. R. astr. Soc.* **172**, 513–533 (1975).
5. Kapahi, V. K. *Mon. Not. R. astr. Soc.* **172**, 513–533 (1975).
6. Speed, B. & Warwick, R. S. *Mon. Not. R. astr. Soc.* (in the press).
7. Colla, G. et al. *Astr. Astrophys. Suppl.* **1**, 281–317 (1970).
8. Colla, G. et al. *Astr. Astrophys. Suppl.* **11**, 291–325 (1973).
9. Bennett, A. S. *Mem. R. astr. Soc.* **68**, 163–172 (1962).

10. Bridle, A. H., Davis, M. M. & Fomalont, E. B. *Astr. J.* **77**, 405–443 (1972).
 11. Warwick, R. S. & Spencer, R. E., *Mon. Not. R. astr. Soc.* (in the press).
 12. Clarke, J. M. & Muxlow, T., *Mon. Not. R. astr. Soc.* (in the press).
 13. Warwick, R. S. *Mon. Not. R. astr. Soc.* **179**, 1–11 (1977).
 14. Pooley, G. G. & Kenderdine, S. *Mon. Not. R. astr. Soc.* **139**, 529–550 (1968).
 15. Pooley, G. G. *Mon. Not. R. astr. Soc.* **144**, 101–127 (1969).
 16. Speed, B. *Mon. Not. R. astr. Soc.* **177**, 137–155 (1976).
 17. Wardle, J. F. C. & Miley, G. K. *Astr. Astrophys.* **30**, 305–315 (1974).

Carbon and M-type giant stars in the Magellanic Clouds

THE late M- and C-type giant stars are of special interest in studies of stellar and galactic evolution as they are found at the extreme low temperature limit which stars first reach after evolving away from the main sequence, and also because they show remarkably different galactic distribution. Because of the pronounced strength of absorption bands of molecular oxides and of the carbon and cyanogen molecules in the spectra of these stars, extremely small dispersions can be used. Modern large telescopes equipped with Ritchey-Cretien optics allow relatively large fields to be surveyed to faint limiting magnitudes. Thus stars can be segregated and classified whose distance moduli are 22 mag and more. Now stellar populations can be sampled and surveys made of the distribution of the stars in space in the Magellanic Clouds and in the less obscured regions near the galactic centre. Three such surveys are described here. They indicate that unsuspected differences in the mixture of C and late M giant stars exist in the nuclear bulge of the galaxy and in various regions of the Magellanic Clouds. Such studies also yield new information concerning the intrinsic luminosities of C and M giant stars in these three galactic systems.

Nassau and Van Albada¹ showed in 1949 how red giant stars could be recognised and classified spectroscopically in the near-infrared region with a dispersion of $1,700 \text{ Å mm}^{-1}$ at the atmospheric A band. Their findings were used to determine the galactic distribution of C- and M-type giants. While these two spectral types show strong concentration towards the galactic equator, the M-giants are found in large numbers toward the galactic nucleus and the C stars towards the anticentre². This suggests that within the galactic nuclear bulge a large concentration of M giants must exist and but few carbon stars.

We have, with Hoag's unpublished data, surveyed the clear nuclear bulge region near the globular cluster

NGC6522 known as Baade's Window. The survey was carried out at the prime focus of the Cerro Tololo Inter-American Observatory's 4-m telescope with the aid of a grating-prism combination of the kind described by Bowen and Vaughan³, and by Hoag⁴. A spectral dispersion of $2,300 \text{ Å mm}^{-1}$ at the A band was achieved. On hypersensitised Kodak IV-N plates, exposed for 60 min, spectra as faint in the near infrared as $m_i \text{ mag} = 18.5$ in the photometric system of Kron, Gascoigne and White⁵ were recorded; this limiting magnitude exceeds by three magnitudes the faintest red giant star found in the survey. The M giants found are so numerous that if one counts only stars of type M6 or later, one reaches a surface density of 2,400 stars per deg^2 . By contrast, C stars are virtually non-existent in the galactic bulge since only one such star appeared in the search area whose diameter is 23 arc min.

In the present investigation, the same observational instrumentation and techniques were used to study the distribution of red giant stars in the large and small Magellanic Clouds (LMC and SMC). In the LMC seven sample 0.12 deg^2 areas were investigated. Of these, three are found within the LMC Bar, including one in the optical centre, one in the so-called 'radio' centre which marks the centre of the LMC neutral hydrogen distribution and one in the western sector of the Bar; the remainder are at various outlying points up to 3.5° away from the optical centre in the northern, northeastern, eastern and southern directions. For the positions of the optical and radio centres, the coordinates published by Bok⁶ were used. The SMC centres will be described later.

The LMC survey is an extension to a fainter apparent magnitude of the earlier survey carried out by Blanco and McCarthy⁷, who used a thin objective prism with the Curtis Schmidt telescope at Cerro Tololo to study the distribution of late M giants over an 8.75 deg^2 area of the LMC. The spectroscopic dispersion of $6,700 \text{ Å mm}^{-1}$ in that study allowed the classification of late M giants into a natural group of stars of about M6.5 and later. In the present survey, it was possible to classify each M star to the nearest half subclass. When stars of type M6.5 and later are segregated they show surface densities that are somewhat higher but still in reasonable agreement with the ones found previously; the differences are probably caused by a different cut-off in spectral types near class M6.5, a problem caused by the extremely small spectroscopic dispersion of the thin-prism survey. As will be discussed later, many C type stars are found in the LMC. These were not detected in the

Table 1 Carbon and late type M Stars in selected regions of the Small and Large Magellanic Clouds and in a central region of the Galaxy

Region	Position R.A. (1975)	Dec. (1975)	C stars	Number M stars	C/M
Small Magellanic Cloud					
SMC central regions					
Bar region	0 h 49.5 min	$-73^\circ 25'$	80	3	26.7
Wing region*	1 h 00.0 min	$-73^\circ 10'$	55	1	55.0
SMC peripheral region					
NGC121 region	0 h 36.6 min	$-71^\circ 55'$	4	4	1.0
Large Magellanic Cloud					
LMC central regions					
O region	5 h 24.5 min	$-69^\circ 48'$	79	37	2.1
R region	5 h 20.2 min	$-68^\circ 55'$	38	23	1.7
Bar-west region*	5 h 08.9 min	$-69^\circ 06'$	71	40	1.8
LMC peripheral regions					
N region	5 h 27.0 min	$-66^\circ 24'$	7	6	1.2
NE region	5 h 47.0 min	$-67^\circ 42'$	8	6	1.3
E region	5 h 47.0 min	$-70^\circ 30'$	32	22	1.5
S region	5 h 24.0 min	$-72^\circ 30'$	5	12	0.4
Baade's Window*	Galactic nuclear bulge region 18 h 02.0 min	$-30^\circ 02'$	1	310	0.003

Area of each region in Table 1 is 0.12 deg^2 .

*Photoelectric sequence has been established in this region.

previous survey although, *a posteriori*, it is possible to recognise them on the thin-prism plates and, in fact, a small fraction (less than 5%) of such C stars were erroneously included in the natural group of late type M giants. Again this is not surprising considering the small dispersion used. An important result of the survey described here is that it confirms the main features of the LMC M-giant distribution by surface density and by brightness found by Blanco and McCarthy, namely, the late M giants are more frequently found in the central regions of the LMC than in the peripheral ones and their magnitude distribution shows a well defined frequency maximum at $m_1 = 14.0$ with a dispersion of ± 0.6 mag. In interpreting their result, Blanco and McCarthy assumed that the late M giants in the solar neighbourhood have an absolute visual magnitude of about -1.0 which, with known $(V-I)$ colours, represents a near infrared luminosity $m_1 = -4.6$, similar to that found for the LMC M giants. However, the previous survey made in Baade's Window suggests strongly that the galactic disk late type giants are actually two or more magnitudes fainter than $M_v = -1.0$. Thus there is a marked luminosity difference between M giants in the LMC and in the galactic nuclear bulge. LMC giants of type M seem to be quite comparable to the red giants found in globular clusters in the Galaxy. Although this result is consistent with early theoretical expectations of Hoyle and Schwarzschild⁸, the precise luminosity to be expected of the red M giants in the LMC cannot now be estimated because we lack precise knowledge of metal abundances of late type stars even in the nuclear bulge of our own galaxy. Now we are for the first time detecting the giant branch of late type stars in nearby galaxies. Abundance data must come later from photometric and higher dispersion spectral surveys in the future.

Three SMC sample areas were also included in the current investigation. One is located in the SMC Bar, another in the Wing and the third in an outlying region near the cluster NGC 121. Table 1 shows the results of the present survey for both Magellanic Clouds. By way of comparison our previous results for Baade's Window are also listed. While C stars are extremely rare in the galactic nuclear bulge, they are found frequently in the Magellanic Clouds especially in the central regions. Ordered according to decreasing metallicity, these regions would be as follows: galactic nuclear bulge, LMC, and SMC. Table 1 reflects the same ordering in the ratio of the number of C stars to the number of late M giant stars but in a reversed sense: almost no C stars are seen in the nuclear bulge where late type M giants abound; C stars outnumber M stars about 2 : 1 in the LMC central areas, while in the SMC practically no M stars are found and the C stars are most frequent in occurrence.

From previous knowledge, the peripheral regions of our galaxy could also be included in this ranking somewhere between the nuclear bulge and the LMC. Although this suggests that chemical composition may be an important determinant of the ratio of C to M giants, age differences must also be taken into account. Some C and M type giant stars have been found⁹ in globular clusters in the Clouds but their number is too small to allow one to make reliable correlations with the metal abundances of the parent globular clusters. Improved theoretical knowledge of cool giant stars will be required before these results can be better interpreted. Nevertheless the present study indicates that if having a C type spectrum is but a stage in the evolution of red giants, as suggested by Wallerstein¹⁰, then the galactic bulge M giants, numerous as they are, must all be approximately coeval and have similar masses and chemical compositions.

Near infrared (m_1) photometry of the C stars was done in the present study, using Kodak I-N plates exposed through a Schott RG 695 filter and newly established photoelectric

near-infrared magnitude sequences. The mean m_1 value for the LMC C stars is 14.0 for the field which contains the photoelectric sequence, LMC Bar-west; for the SMC the corresponding value is 14.6 m_1 (in the SMC Wing region which also has a photoelectrically determined magnitude sequence). The difference is that to be expected from the distance moduli of the two Magellanic Clouds. In both clouds, then, the near infrared luminosities for C stars is $M_1 = -4.6$, similar to the M giants as found above. Earlier reliable estimates of the luminosities of C stars are lacking, but Gordon's study¹¹ suggests that no marked difference exists with the luminosity of the carbon stars in the solar neighbourhood.

The frequency of C stars in the central or bar regions of the two clouds is remarkably high, about 600 stars per deg.². This is another indicator that the clouds have had rather different evolutionary histories from the galaxy.

Comparing the Table 1 data for the LMC and SMC late type giants one finds the SMC extremely deficient in late type M giants. Also, stars as late as M9 are found in the direction of the galactic centre, stars as late as M8 are found in the LMC but in the SMC no M star later than M6 has been found. Detailed lists of the stars found in this study including photometric and positional data will be published elsewhere.

Earlier studies of the red giants in the LMC but to a much brighter limiting magnitude were made by Westerlund and coworkers^{12,13}; these did not apparently detect the main features of the distribution of red giants outlined here, although undoubtedly the very brightest of the C stars and the M supergiants were recognised and recorded. More recently Sanduleak and Davis Philip¹⁴ have surveyed the C stars in the SMC with objective prism spectra obtained in the spectral region of the C₂ Swan band using the Curtis Schmidt telescope. The overall distribution found by them seems to be correct but their survey shows only a small fraction of the stars detected in the current investigation. Sanduleak provided us with a list of the positions of his SMC carbon stars so that we could compare results.

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B. M. BLANCO
V. M. BLANCO

Cerro Tololo Inter-American Observatory,
La Serena, Chile

M. F. MCCARTHY

Vatican Observatory,
Vatican City State

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1. Nassau, J. J. & Van Albada, G. B. *Astrophys. J.* **109**, 391 (1949).
2. Blanco, V. M. *Galactic Structure* (eds Blaauw, A. & Schmidt, M.) **5**, 241 (University of Chicago Press, Chicago, 1965).
3. Bowen, I. S. & Vaughan, A. H. *Publs astr. Soc. Pacif.* **85**, 174 (1973).
4. Hoag, A. A. *Publs astr. Soc. Pacif.* **88**, 860 (1976).
5. Kron, G. E., Gascoigne, S. C. B. & White, H. S. *Astr. J.* **62**, 205 (1975).
6. Bok, B. J. A. *Rev. astr. Astrophys.* **4**, 125 (1966).
7. Blanco, V. M. & McCarthy, M. F. *Nature* **258**, 407 (1975).
8. Hoyle, F. & Schwarzschild, M. *Astrophys. J. Suppl. Ser.* **2**, 1 (1955).
9. Feast, M. & Evans, T. L. *Mon. not. R. astr. Soc.* **164**, 152 (1973).
10. Wallerstein, G. A. *Rev. astr. astrophys.* **11**, 115 (1973).
11. Gordon, C. P. *Astrophys. J.* **153**, 915 (1968); *Publs astr. Soc. Pacif.* **80**, 597 (1968).
12. Westerlund, B. E. *Uppsala Ann.* **4**, 7 (1960); *The Galaxy and the Magellanic Clouds*, IAU-URSI Symposium No. 20 (eds Kerr, F. J. & Rogers, A.W.) 239 (Australian Academy of Sciences, Canberra, 1964).
13. Crabtree, D. R., Richer, H. B. & Westerlund, B. E. *Astrophys. J. Lett.* **203**, L 81 (1976).
14. Sanduleak, N. & Davis Philip, A. G. *Bull. Am. astr. Soc. Abstr.* **8**, 537 (1976).

How to locate the electrically conducting fluid core of a planet from external magnetic observations

A NEW method is proposed here for locating the electrically conducting fluid core of a planet from external magnetic observations. When tested with the aid of geomagnetic data, the method gives for the radius of the Earth's liquid metallic core a value which differs insignificantly from the accepted value (based on seismic data). These findings have important implications for future investigations of the magnetic fields and internal structure and dynamics of the Earth, Jupiter, and other magnetic planets.

Consider, as a good first approximation to a real planet in the present context, an isolated spherical planet of radius r_s consisting of an electrically conducting spherical fluid core of conductivity σ and radius r_c surrounded by a non-conducting mantle of thickness $r_s - r_c$. Suppose that the planet is embedded in a non-conducting atmosphere which extends to infinity and that the whole of space $r > 0$ is pervaded by a magnetic field $\mathbf{B}(\mathbf{r}, t)$ due entirely to electric currents of density $\mathbf{j}$ flowing in the conducting core. Any effects due to permanent magnetism are assumed negligible so that the magnetic permeability μ is everywhere equal to that of free space. (Here t denotes time and $\mathbf{r}$ is the vector distance of a general point from the centre of the planet.)

Specifying $\mathbf{j}$ in $0 < r < r_c$ suffices to determine $\mathbf{B}$ throughout the whole of the planet and the surrounding space, but the inverse problem of determining $\mathbf{j}$ in $0 < r < r_c$ from a full description of $\mathbf{B}$ outside the core has no unique solution. Nevertheless, as we shall argue later, measurements at any epoch t of $\mathbf{B}(\mathbf{r}, t)$ and also of its time rate of change $\partial\mathbf{B}/\partial t$ over any closed surface that encloses the planet can be used to determine the radius r_c of the core of the planet in a simple and straightforward way. This is so provided that measurable fluctuations in $\mathbf{B}$ occur on time scales τ that are so short in comparison with the time scales on which effects due to ohmic decay make themselves felt that the fluctuations can, with sufficient accuracy, be ascribed largely to the rearrangement of lines of magnetic force by fluid motions in the core.

The last condition requires that

$$\tau \equiv \overline{B/\partial B/\partial t} < \mu \sigma \alpha^2 r_c^2 \quad (1)$$

where $\overline{B}$ and $\overline{\partial B/\partial t}$ are typical magnitudes of $\mathbf{B}$ and $\partial\mathbf{B}/\partial t$, respectively, and αr_c is a length-scale characteristic of the spatial distribution of $\mathbf{j}$ in $r < r_c$, α being typically ≤ 1 . Over time intervals satisfying equation (1) (which are, of course, very much less than the time scale associated with the generation of the magnetic field by the dynamo process) we can, to a first approximation, treat the core as a perfect electrical conductor, for which it is impossible, by Faraday's law of electromagnetic induction, to change the magnetic flux linkage $N(r = r_c)$ (ref. 1, for further details and references see refs 2 and 3). (Fluctuations in $N(r = r_c)$ when σ is infinite would generate electric currents of infinite strength, requiring an infinitely powerful source of energy.) For any fixed closed (but not necessarily spherical) surface Σ given by $r = R$ which encloses the origin $r = 0$ we can define a quantity $N(t; r = R)$ equal to the total number of intersections of lines of magnetic force with Σ ; thus

$$N(t; r = R) \equiv \iint_{\Sigma} |\mathbf{B} \cdot d\mathbf{A}| \quad (2)$$

$d\mathbf{A}$ denotes the vector element of area of Σ , over the whole of which the area-integral is taken. At the bounding surface of a perfect conductor, N is independent of t , so that $\delta N/\delta t$ vanishes and so do all higher derivatives $\delta^2 N/\delta t^2$ and so on.

The quantity N can be calculated quite simply (in principle) for all surfaces Σ that lie outside the core, as $\mathbf{j} = 0$ in $r > r_c$ and $\mathbf{B}$ in $r > r_c$ can therefore be expressed as the gradient of a scalar V (say) satisfying Laplace's equation $\nabla^2 V = 0$. This property enables $\mathbf{B}$ to be determined everywhere in $r > r_c$ from measurements of $\mathbf{B}$ over any single closed surface in that region, such as the accessible surface of the planet in $r = r_s$ or a closed surface effectively covered by an orbiting satellite carrying a magnetometer. Thus, this new method for determining the radius r_c of the electrically conducting core of a planet from measurements of $\mathbf{B}$ in $r \geq r_s$ involves, in principle, taking such measurements for two epochs $t = t_1$ and $t = t_2$ separated by a time interval $t_2 - t_1$ satisfying the inequality expressed by equation (1), and calculating from these measurements the quantity

$$\Delta N(t_1, t_2; r = r_s - p \Delta r) \equiv N(t_2; r = r_s - p \Delta r) - N(t_1; r = r_s - p \Delta r) \quad (3)$$

at successive levels $r = r_s - p \Delta r$ within the planet, where $p = 0, 1, 2, 3, 4$, and so on, marching downwards from the surface in conveniently short steps of length Δr . In general, ΔN will be non-zero when $p = 0$ (corresponding to the surface of the planet $r = r_s$) and also at successive values of p , if Δr is small enough, until p reaches that value p_c (say), at which ΔN effectively vanishes. The value of r_c is then given by

$$r_c = r_s - p_c \Delta r \quad (4)$$

To avoid ambiguity, it is necessary to find all values of p at which ΔN is equal to zero and then select the correct value of p_c by requiring also that higher time-derivatives of N should vanish.

Further details of the method, including the systematic analysis of the physical and mathematical approximations upon which it is based, and the treatment of errors will be reported elsewhere (R.H., F. J. Lowes & S. R. C. Malin, in preparation). It is enough to remark here that the first practical result based on the new method is encouraging (if it is not fortuitous), for when applied to the Earth, for which $r_s = 6,371$ km, the method gives for r_c a value which differs insignificantly, by less than 2%, from the accepted value $3,486 \pm 5$ based on seismological data. (This calculation was kindly carried out by S. R. C. Malin and colleagues at the Geomagnetism Unit of the Institute of Geological Sciences, NERC, using one of the best available models of the geomagnetic secular variation, where determinations of the main geomagnetic field—namely that field obtained when contributions due to ionospheric and magnetospheric currents have been removed by taking annual mean values of the observations—for the period 1965 to 1975 were fitted by a spherical harmonic series up to and including terms of degree 8.) The method should prove valuable in the investigation of the internal structure of other magnetic planets, such as Jupiter⁴, Saturn and Mercury (for which the sizes of the electrically conducting fluid cores are not yet known), provided that sufficiently detailed measurements of their main magnetic fields and secular changes can be made in future work with space-probes. If the pressure at the level $r = r_c$ in Jupiter is equal to the critical pressure P at which molecular hydrogen changes to its metallic form, which is several megabars and well above the range of operation of high pressure laboratory apparatus, then the determination of r_c by the proposed new method will lead directly to an estimate of P which would serve as a check on estimates¹ based on shock wave experiments and quantum mechanical calculations. On the other hand, if r_c is so close to r_s that the corresponding pressure at $r = r_c$ is well below present estimates of P (even allowing for the large uncertainties in these estimates, more than a factor of two), then it would be necessary to take seriously the possibility (see refs 4 and 5) that impurities render the molecular hydrogen layer in Jupiter sufficiently conducting to support dynamo action within it.

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RAYMON HIDE

*Geophysical Fluid Dynamics Laboratory,
Meteorological Office,
Bracknell, Berkshire, UK*

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1. Bondi, H. & Gold, T. *Mon. Not. R. astr. Soc.* **110**, 607–611 (1950).
2. Backus, G. E. *Phil. Trans. R. Soc. Lond.* **A263**, 239–266 (1968).
3. Jacobs, J. A. *The Earth's Core* (Academic, London, 1975).
4. Gehrels, T. (ed.) *Jupiter: Studies of Interior, Atmosphere, Magnetosphere and Satellites* (University of Arizona, Tucson, 1976).
5. Hide, R. in *Magnetism and the Cosmos* (eds. Hindmarsh, W. R., Lowes, F. J., Roberts, P. H. & Runcorn, S. K.) 378–393 (Oliver and Boyd, Edinburgh, 1965).
6. Elphic, R. C. & Russell, C. T. *Geophys. Res. Lett.* (in the press, 1978).

ULF tree potentials and geomagnetic pulsations

HIGH-SENSITIVITY measurements of ultra-low-frequency (ULF; frequencies less than 5 Hz) geomagnetic pulsations¹ usually require elaborate receiving antennas ranging from large air-cored coils² through multi-turn steel, or mumetal-cored solenoids^{3–5} to small superconducting loops immersed in liquid helium⁶. Pairs of electrodes inserted in the ground have also been used as antennas^{7,8}. The need for a large spacing between the electrodes (varying from hundreds to thousands of metres) and the difficulty of calibrating the measurements absolutely have resulted in the almost universal use of the more compact and easily calibrated coil-type antennas in recent years. I describe here a new method for measuring ULF geomagnetic pulsations, which requires a minimum of elaborate equipment. The method is based on the use of trees, or, more specifically, on the use of pairs of electrodes inserted into trees, as ULF receiving antennas.

There are several reasons that this new method of measurement may be of interest. The equipment is simple and thus the method could lead to more widespread observations of ULF geomagnetic pulsation phenomena. The method of measurement also provides new information about tree potentials, that is, it shows that some, and perhaps all, of the ULF components of these potentials are induced by ULF geomagnetic field fluctuations and do not originate in the trees themselves. Finally, although it is not clear at present what effect induced ULF electric fields may have on the growth and other vital processes in a tree, the link between these ULF electric fields and geomagnetic field fluctuations suggests that some environment-related changes in trees could also be influenced by changes in geomagnetic activity. These changes may have a natural origin (for example, the changes that occur during a solar cycle⁹) or they may be caused by a variety of human activities (by modern d.c.-powered mass transit systems, which can produce large amplitude ULF electromagnetic fields¹⁰).

The ULF measurements reported here were stimulated by the work of Burr on relatively steady-state tree potentials¹¹. Burr recorded these potentials for more than a decade using a pair of specially-designed non-polarisable electrodes inserted in the cambium of an unspecified tree (which was probably a maple). The electrodes were about a metre apart along the long axis of the tree and Burr observed diurnal, 27-d, and seasonal variations, as well as a suggestion of a correlation with sunspot activity, in their potential difference.

Most of Burr's observations were at frequencies far below the frequency range for ULF geomagnetic pulsations. One series of measurement obtained, however, during an electrical storm suggested that ULF variations of tree potentials might occur on occasion. I therefore began a search for variations with frequencies predominantly in the

Pc 1 geomagnetic pulsation range (0.2–5 Hz). These frequencies correspond approximately to the delta regime for human brain waves.

The measurements were made using a large native oak, *Quercus lobata*, that was located near conventional ULF recording equipment at a site on the Stanford University campus. This latter equipment uses 20,000 turn steel-cored solenoids as ULF antennas and it operated continuously throughout the interval during which the tree measurements were made. Thus, simultaneous measurements of ULF geomagnetic pulsations using both conventional loop antennas and a tree 'antenna' were obtained at the one location.

Two steel nails were used as electrodes. Following Burr's configuration, they were inserted about 0.05 m into the tree along the long axis, with a spacing of 0.76 m. The lower electrode was approximately 1 m above the ground, and the two electrodes faced toward the geomagnetic west. Because the tree was not completely vertical, a line joining the two electrodes would have been inclined approximately 20° toward the geomagnetic east. The diameter of the tree midway between the two electrodes was 0.65 m.

A resistance of about 5 k Ω was typically observed between the electrodes, increasing to about 10 k Ω if polarisation was allowed to occur. A d.c. potential difference was also observed that varied from day to day but whose absolute value was usually in the range 10 to 100 mV, with the upper electrode positive. The electrodes were connected to a low-frequency high-gain amplifier through an RC filter ($R=22$ M Ω , $C=50$ μ F). The amplifier was usually set for 50 db gain, and its output was filtered (0.02–7 Hz) before being recorded, generally without additional amplification, on a chart record and on analog magnetic tape.

The ULF signals measured by this system were undoubtedly induced in the tree 'antenna' and not in the shielded cabling between the electrodes and the recording system: when the electrodes were disconnected from the tree and connected to an equivalent 5 k Ω resistor, without any other change in the wiring or configuration of the system, only a steady low level of white noise (typical resistor thermal noise) was observed.

Similarities between the ULF signals recorded conventionally and with the tree 'antenna' were immediately apparent on the chart records. More detailed analysis confirmed that Pc 1 pulsation events recorded by the two systems were very nearly identical in all their important characteristics. Figure 1, for example, shows spectrograms of a sequence of four Pc 1 pulsation events that occurred during the interval 1200 to 1500 UT on 17 January 1976, and which were received by the tree 'antenna' (a) and the conventional north-south solenoid antenna (b). With the exception of a lower signal-to-noise ratio for the tree measurements, the two Pc 1 pulsation records are closely alike. It will also be noticed that the lower frequency Pc 2/Pc 3 geomagnetic activity (frequencies in the range 0.02 to 0.2 Hz) is recorded similarly by both systems. The amplitude of the ULF pulsations in the tree potentials is very small. For the Pc 1 pulsations shown in Fig. 1, the maximum amplitude of the potential fluctuations was about 0.1 mV.

The nearly identical occurrence and spectral characteristics of ULF events measured by the tree electrodes and by the conventional ULF equipment indicated that the tree potentials were largely induced by ULF time variations of the geomagnetic field. To investigate this possibility, a portable planar search coil powered by a 1 Hz signal generator was moved around the tree near the electrodes. It was found that a 1 Hz oscillation of the potential difference between the tree electrodes was produced only when the search coil was orientated with its moment vector in the north-south direction. When the two electrodes were moved to the north face of the tree, a response from the electrodes could be obtained only when the search coil moment vector was orientated in the east-west direction.

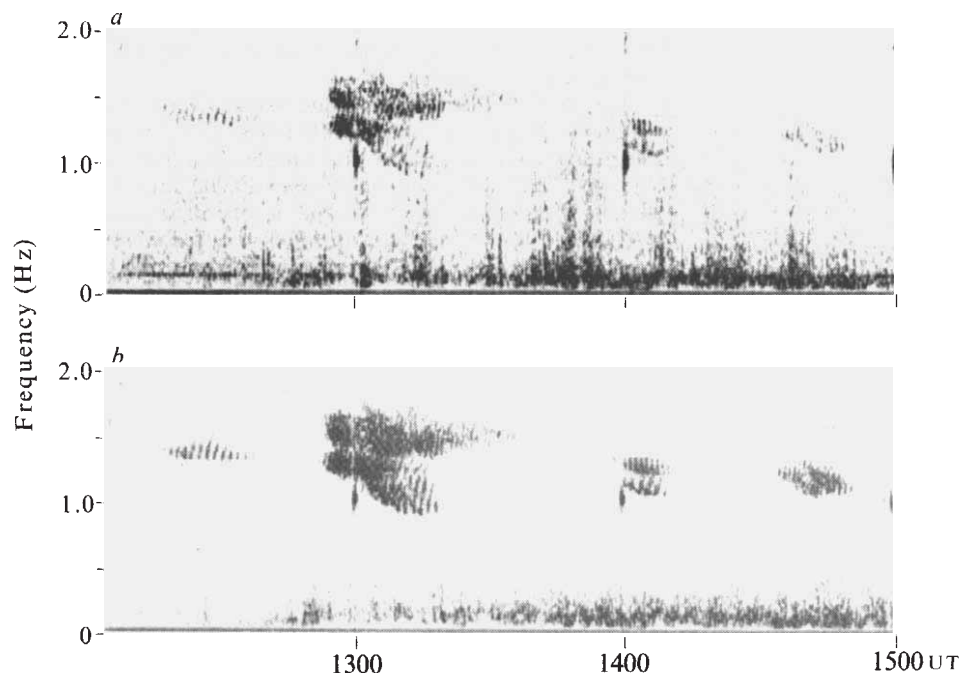


Fig. 1 Spectrograms of a series of Pc 1 geomagnetic pulsation events recorded at Stanford, California, using tree potentials (a) and a conventional solenoid antenna (b). Short intervals of a 1 Hz calibration signal appear at the start of each hour. The vertical lines in the upper spectrogram are caused either by local electromagnetic transients or by natural sferics; similar lines occur in the lower spectrogram, but they are not as obvious because the background noise is comparatively suppressed.

These results, and the observations of natural Pc 1 pulsations, can possibly be best understood by considering the tree/electrode pair combination to form a collection of conducting loop antennas in which e.m.f.s may be induced by magnetic field fluctuations in the appropriate direction. The conducting paths are provided by the conducting material of the tree (and the cambium in particular¹¹), and, for field fluctuations in a particular direction, the area of the relevant loop antenna is defined by the intersection of the tree with a vertical plane perpendicular to the particular field direction and passing through the two electrodes. Thus, in the measurements reported here, the Pc 1 pulsation events observed in the tree potentials were produced by Pc 1 pulsations of the north-south component of the geomagnetic field.

Further tests showed that the tree potentials could only be detected in a living tree. Thus, when a tree dies, the potentials gradually disappear as the wood dries and loses its conductivity.

In conclusion, measurements with tree electrodes show that trees may be used as 'antennas' to detect ULF geomagnetic pulsations. The measurements also show that ULF tree potentials are largely produced by ULF fluctuations of the geomagnetic field (the remaining component of the potentials is probably thermal noise). Presman¹² noted that electromagnetic fields usually have an adverse effect on living processes. If the ULF geomagnetic pulsations have any adverse effect on the growth of trees (and, as we have seen, they must induce electric currents in the living material) these effects could possibly be observed in tree ring data. Pc 1 geomagnetic pulsation occurrences vary markedly over a solar cycle⁹ and thus, if these particular pulsations effect tree growth, a solar cycle in tree ring data could occur. LaMarche and Fritts¹³ searched unsuccessfully for a relation between tree ring data and sunspot numbers. The phase of the Pc 1 pulsation solar cycle, however, differs by several years from the sunspot cycle and, assuming the two cycles affect tree ring data, they may tend to obscure each other's effects. Furthermore, other geomagnetic pulsations and higher-frequency electromagnetic signals have their own cycles of occurrence, and their effects on tree ring formation, if any, could add further to the complexity of the tree ring data. Studies of these possible effects are desirable, because the tree ring data could provide a unique

record of past ULF and higher-frequency geomagnetic activity.

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A. C. FRASER-SMITH

*Radioscience Laboratory,
Stanford Electronics Laboratories,
Stanford University,
Stanford, California 94305*

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1. Jacobs, J. A. *Geomagnetic Micropulsations* 15 (Springer, New York, 1970).
2. Campbell, W. H. *Proc. IEEE* **51**, 1337-1342 (1963).
3. Tepley, L. R. *J. geophys. Res.* **66**, 1651-1658 (1961).
4. Lokken, J. E., Shand, J. A. & Wright, C. S. *J. geophys. Res.* **68**, 789-794 (1963).
5. Lokken, J. E. in *Natural Electromagnetic Phenomena Below 30 Kc/s* (ed. Bleil, D. F.) 373-428 (Plenum, New York 1964).
6. Buxton, J. L. & Fraser-Smith, A. C. *IEEE Trans. Geosci. Elec.* **GE-12** 109-113 (1974).
7. Troitskaya, V. A. *J. geophys. Res.* **66**, 5-18 (1961).
8. Heacock, R. R. & Hessler, V. P. *J. geophys. Res.* **67**, 3985-3995 (1962).
9. Fraser-Smith, A. C. *J. geophys. Res.* **75**, 4735-4745 (1970); *J. geophys. Res.* **77**, 4209-4220 (1972).
10. Fraser-Smith, A. C. & Coates, D. B. *Radio Sci.* (submitted).
11. Burr, H. S. *Yale J. Biol. Med.* **17**, 727-734 (1945); *Yale J. Biol. Med.* **19**, 311-318 (1947); *Science* **124**, 1204-1205 (1956).
12. Presman, A. S. *Electromagnetic Fields and Life* (transl. Sinclair, F. L., ed. Brown, F. A. Jr) 155 (Plenum, New York, 1970).
13. LaMarche, Jr, V. C. & Fritts, H. C. *Tree-Ring Bull.* **32**, 19-33 (1972).

Mass spectrometric measurement of the positive ion composition in the stratosphere

THE ion composition of the upper atmosphere has been the subject of many experiments. Early mass spectrometer measurements have been primarily concerned with the ion composition at altitudes between 60 and 250 km (refs 1-6). Few data, however, are available on the charged particle composition of the stratosphere. Global ion density measurements using electric probes⁷⁻¹¹ indicate number densities of the order of 100-10,000 cm⁻³ for positive ions in this region. Arnold *et al.*¹² have recently published the first mass spectrometric data of the stratospheric ion composition, obtained on the downleg portion of three rocket flights. Above 40 km they observe the proton hydrates as being the most abundant ions. Below 40 km a change in the predominant ion species is seen, which is explained by Arnold *et al.* by ion molecule reactions between water cluster ions and

formaldehyde. One of the possible dangers, inherent to rocket flights, however, is the alteration of the ion composition, due to shock wave-induced fragmentation. Mass spectrometric measurements on this region using a balloon-borne instrument were suggested¹³ as early as 1969. Here we give the first preliminary results of a successful flight, performed with a balloon-borne quadrupole mass spectrometer on 30 September 1977. A 100,000 m³ Zodiac balloon was flown at mid-latitude (CNES launching site at Aire sur l'Adour, France, 44°N) at 18.27 UT and reached the altitude of 35 km at 20.00 UT. The measurements were started at 20.14 UT, which is after sunset.

The gondola, which will be described in more detail elsewhere, primarily consists of a high-speed helium cryopump, in which is built a quadrupole mass filter, followed by a high gain electron multiplier. The ions were sampled through a 0.2 mm hole in a 0.1 mm thickness stainless steel flange, which can be biased with respect to the gondola structure. Signal detection is realised

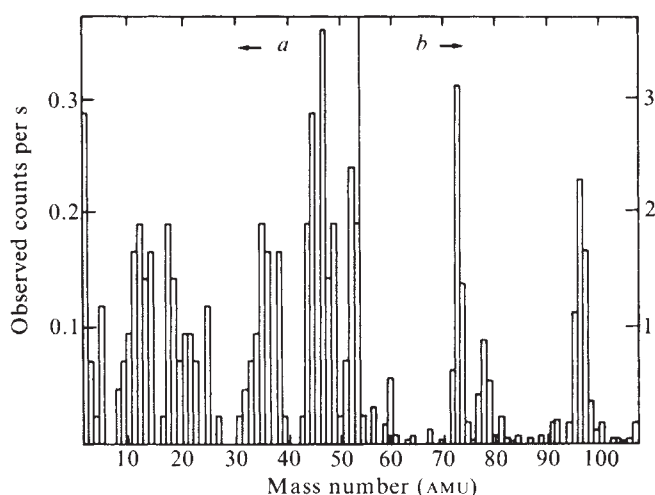


Fig. 1 Observed count rate versus mass number measured at 35 km altitude. Both *a* and *b* have been obtained by averaging of the counts observed, during seven scans. The applied draw-in-potential was -13 V.

by both analogue and pulse counting techniques. The mass spectrometer covers the mass range from 0 to 109 AMU. Measuring in mass domains as well as sweeping the complete mass range is possible¹¹. Sweeping the complete mass range was realised by using discrete steps approximately 1 AMU in size. The integration time for pulse counting was 6 s per step. Thus spectra were obtained in the form of a histogram, as is shown in Figs 1 and 2. A fixed resolution mode was used throughout all scans reported here. Two separate draw-in-potentials were used, -13 and -4.6 V. Figure 1 represents the results obtained at -13 V and Fig. 2 those at -4.6 V. It should be noted that in both figures the number of observed counts per second for masses smaller than 55 AMU have been represented with a different scale factor. The count rate due to noise pulses was smaller than 0.1 s^{-1} during the whole flight.

The mass numbers of the detected ions are listed in Table 1, with the draw-in-potentials and the uncertainty in atomic mass numbers. Counts below mass 10 have not been taken into account, because instabilities have been observed in the power supply of the quadrupole for such low mass values.

As can be seen from Table 1, the uncertainty for lower mass numbers is rather high for two reasons. First, the fixed resolution, chosen rather low in favour of high sensitivity, gives rise to much more broadened peaks at lower masses, than at higher ones. Second, due to the low counting rate at low mass numbers, it was impossible to build up a complete histogram even after several scans and thus a faulty conclusion may be drawn about

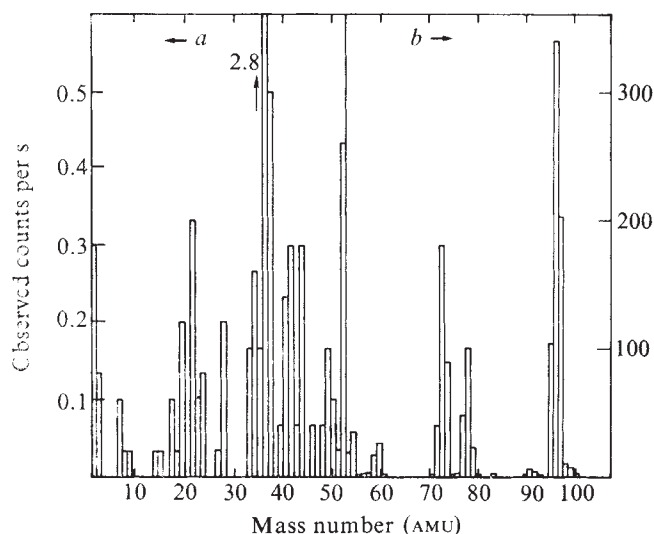


Fig. 2 Observed count rate versus mass number at 35 km altitude. *a*, The result of five scans; *b*, two scans. Applied draw-in-potential was -4.6 V.

the exact position of the pass peaks. Above mass 50, however, the signal was strong enough to permit a higher degree of certainty. Furthermore, the mass domain from 50 to 109 AMU has been scanned with a higher resolution and the results confirm those of Table 1. Masses 19 (18 ± 3 and 20 ± 3), 37, 55, 73 and 109 can be identified with the cluster ions $\text{H}^+(\text{H}_2\text{O})_n$, n ranging from 1 to 6.

In all spectra, mass 73 was the most abundant peak among the water cluster ion peaks. The observed distribution of the $\text{H}^+(\text{H}_2\text{O})_n$ cluster ion peaks is represented in Table 2.

Table 2 does not represent a correct number density distribution of the hydrated hydronium ions, as a correction factor has to be taken into account to convert peak height to number density (even on relative scale). The result of this correction, which is not known exactly and which cancels the effect of lower transmission of the mass filter at higher mass numbers, will be a larger percentage of mass 91. As possible cluster breaking up due to electric fields must also be considered, our results are difficult to compare with the distribution calculated by Mohnen¹⁵.

It is not completely clear why the distribution of $\text{H}^+(\text{H}_2\text{O})_n$ is different at different draw-in-potentials, although again electric field-induced breaking up of the cluster ions may have an important role.

Apart from the water cluster ions, which are expected according to the known reaction schemes¹⁶, additional peaks

Table 1 Observed mass numbers

Draw-in-potential		
-13 V		-4.6 V
13 ± 3		—
18 ± 3		20 ± 3
25 ± 3		—
—		29 ± 3
37 ± 3		37 ± 2
—		43 ± 3
47 ± 3		50 ± 3
55 ± 2		55 ± 2
60 ± 2		60 ± 2
73 ± 2		73 ± 2
78 ± 2		78 ± 2
91 ± 2		91 ± 2
96 ± 2		96 ± 2
109 ± 2		—

Table 2 Observed distribution of $\text{H}^+(\text{H}_2\text{O})_n$ mass peaks at 35 km in %

<i>n</i>	Draw-in-potential		
	Mass number	-15 V	-4.6 V
2	37	5	1
3	55	6	16
4	73	79	80
5	91	5	3
6	109	5	—

have been observed, among which 96 ± 2 was the most abundant. At low draw-in-potentials, this peak was even larger than the one at mass 73. Masses 29, 42, 60 and 80 have also been observed by Arnold *et al.*¹², but they do not mention mass 96 ± 2 , nor do they report that they have observed masses 73, 91 and 109. Because this (96 ± 2) is the most abundant mass peak among the non-proton hydrates which we have observed, it is tempting to conclude that rocket-borne measuring devices are more disturbing than balloon-borne instruments. However, care must be taken, as the ion-molecule reaction channels might be different during night time. The observed mass peaks can be partly fitted into the formaldehyde ion chemistry if 47 ± 3 and 50 ± 3 are tentatively identified as $\text{H}^+(\text{CH}_2\text{O})\text{H}_2\text{O}$ and 96 ± 2 as $\text{H}^+(\text{CH}_2\text{O})_2(\text{H}_2\text{O})_2$ or $\text{C}_2\text{H}_2\text{O}^+(\text{H}_2\text{O})_3$. But, one should be careful because although the existence of formaldehyde in the stratosphere has been predicted theoretically¹⁷, to our knowledge no experimental observation of it has been published. Furthermore, no reaction rate constants of formaldehyde ions or molecules with proton hydrates have been published. Thus any tentative identification must be regarded as speculative. As an alternative interpretation, however, mass numbers 60, 78 and 96 can be tentatively assigned to the hydrates of N_3^+ , namely $\text{N}_3^+(\text{H}_2\text{O})$, $\text{N}_3(\text{H}_2\text{O})_2$ and $\text{N}_3^+(\text{H}_2\text{O})_3$, respectively, which have already been observed in laboratory air flow discharges by Hayhurst and Padley¹⁸. In view of this, 47 ± 3 and 50 ± 3 may be interpreted as due to O_3^+ .

We need more data, especially higher resolution mass spectra and conclusive laboratory work, before any final conclusions can be made. It is clear, however, from the observations reported here and from those of Arnold¹², that apart from the hydrated hydronium ions some other unknown ionic species are present in the stratosphere. This should give a new motivation for more extended laboratory and *in situ* investigations on the ionic chemistry of the stratosphere.

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E. ARIJS
J. INGELS
D. NEVEJANS

Belgian Institute for Space Aeronomy,
3 Ringlaan, B-1180 Brussels, Belgium

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- Johnson, C. Y. *Ann. Geophys.* **17**, 100–108 (1961).
- Istomin, V. G. *Proc. Int. Space Sci. Symp.* **3rd** 209–220 (1963).
- Istomin, V. G. & Pokhunov, A. A. *Proc. Int. Space Sci. Symp.* **3rd** 117–131 (1963).
- Narcissi, R. S. & Bailey, A. D. J. *Geophys. Res.* **70**, 3687–3700 (1965).
- Narcissi, R. S., Bailey, A. D., Wlodyka, L. F. & Philbrick, C. R. *J. atmos. terr. Phys.* **34**, 647–658 (1972).
- Krankowsky, D., Arnold, F., Wieder, H. & Kissel, J. *Int. J. Mass Spectrom. Ion Phys.* **8**, 379–390 (1972).
- Hale, L. C., Hoult, D. P. & Baker, D. C. *Scient. Rep. No. 223*, Pennsylvania State Univ. (1967).
- Mitchel, J. D., Hale, L. C., Olsen, R. O., Randawa, J. S. & Rubio, R. *Aeron. Rep.* **48** (1972).
- Pedersen, A. & Kane, A. J. in *Mesospheric Models and Related Experiments* (ed. Fiocco) 274–278 (Reidel, Dordrecht 1971).
- Smirnykh, L. N. *Cosmic Res.* **14**, 145–148 (1976).
- Rose, G., Widdel, H. U., Azcarraga, A. & Sanchez, R. *Planet. Space Sci.* **20**, 871–876 (1972).
- Arnold, F., Krankowsky, D. & Marien, K. H. *Nature* **267**, 30–32 (1977).
- Ackerman, M. *et al. Aeronomica Acta C* **24** (1969).
- Arijs, E. & Nevejans, D. *Rev. Scient. Instrum.* **46**, 1010–1015 (1975).
- Mohren, V. A. *Pure appl. Geophys.* **84**, 141–153 (1971).
- Ferguson, E. E. in *The Natural Stratosphere of 1974*, CIAP Monograph I, 542–554 (1974).
- Stewart, R. W. & Hoffert, M. I. *J. atmos. Sci.* **32**, 195–210 (1975).
- Hayhurst, A. N. & Padley, P. J. *Trans. Faraday Soc.* **63**, 1620–1630 (1967).

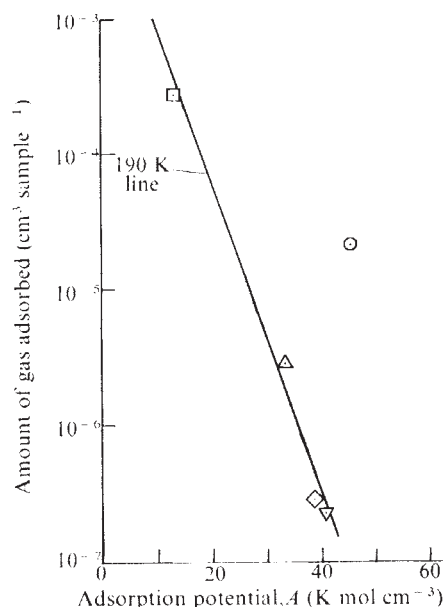
Chemical interpretation of Viking Lander 1 life detection experiment

PRELIMINARY results of the Viking Lander 1 (VL-1) biology experiments¹ revealed that humidification of the martian soil sample in the gas exchange experiment (GEX) released substantial amounts of carbon dioxide and oxygen, as well as detectable amounts of nitrogen and argon or carbon monoxide. We have reviewed the available flight data and found that, when the amounts of evolved gases were plotted in an adsorption potential plot, the amount of evolved oxygen was anomalously high compared to the other gases. This paper also describes simulation experiments with a model Mars soil provided by the Viking Inorganic Analysis Team (ICAT) and treated with a radiofrequency (RF) glow discharge in a simulated martian atmosphere. The findings indicated that the GEX simulation procedure released oxygen, carbon dioxide, and nitrogen in amounts comparable to that seen in the experiment on Mars.

The design of the GEX and its modes of operation have been described elsewhere². The values first reported¹ have been revised, and the maximum amounts of gases evolved from the entire 1 cm³ of humidified sample in the test cell have been given by Oyama *et al.*³ as 83 nmol N₂, 775 nmol O₂, 9,800 nmol CO₂, and 13 nmol Ar/CO. Ar and CO were not separated in the chromatographic column.

The postulate considered here for the gas evolved in the VL-1 humid mode GEX was that the amount of gas seen could have been physically or chemically adsorbed, or attached in a surface complex, on the martian soil. Using a molecular area of 14.6×10^{-20} m² for O₂ and an estimated bulk density of soil sample of 1.3 g cm⁻³, it can be calculated that the evolved O₂ would cover an area of 0.052 m² g⁻¹ soil. The estimated bulk density was the same value as that used in ref. 3 and was the midpoint of the range estimated in ref. 4 from the observed physical behaviour of the fine-grained materials in the immediate vicinity of VL-1. A similar calculation for the CO₂, using a molecular area of 17×10^{-20} m², gives a surface coverage 0.77 m² g⁻¹. A specific surface area of 1 m² g⁻¹ or greater could, therefore, have accommodated the evolved O₂ and CO₂.

Fig. 1 Adsorption potential plot of gas evolved in VL-1 GEX humid mode experiment. The 'A value' ordinate includes sample temperature, *T*, in K, molar volume of liquid adsorbate, *V_m*, in cm³ g⁻¹, and the reciprocal of the relative vapour pressure of the adsorbate, *P₀/P* where $A = (T/V_m) \log_{10}(P_0/P)$. ◇, CO; ○, O₂; □, CO₂; △, N₂; ▽, Ar.



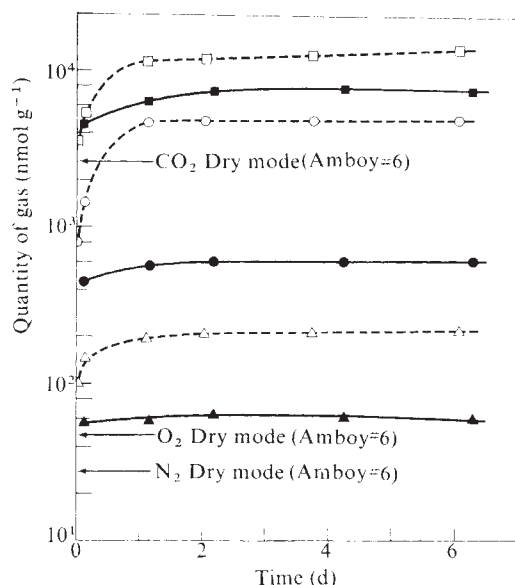


Fig. 2 Gas released in the humid mode from the VL-1 GEX (solid line) and from RF glow discharge treated Amboy no. 6 soil (dashed line). RF glow discharge conditions were 33 mm³ STP s⁻¹ O₂ flow, 0.50 mm Hg = 66 Pa O₂ pressure, 50 W RF power at 13.56 MHz, 398 K sample temperature, and 18 h = 65 ks treatment time (d × 86.4 = ks) ●, ○, ■, CO₂; ▲, △, N₂.

Data for the gas composition of the martian atmosphere⁵ and the equilibrium vapour pressures of the pure gases⁶ were used to calculate the relative pressure, P/P_0 , of the gases in the martian atmosphere at a soil acquisition temperature of 190 K (ref. 7). An adsorption potential plot, similar to that used for the prediction of adsorption of trace gas species in spacecraft contaminant control systems⁸, is shown in Fig. 1. The CO₂, N₂, CO, and Ar points approximate a straight line, but the O₂ point is anomalously high. An adsorbed monolayer volume was derived from the potential plot by extending the straight line to the 'A value' range for physical adsorption of nitrogen at the temperature of boiling liquid nitrogen, and plotting the extrapolated adsorption data as a BET plot. A surface area of 17 m² g⁻¹ was calculated for the martian soil, from a nitrogen molecular area of 16.2 × 10⁻²⁰ m².

A simulated martian soil, Amboy no. 6, had been obtained by ICAT from the Amboy volcanic crater in southern California. It was chosen as a simulated martian soil because of its low organic and high iron content. More recently, synthetic mineral mixtures have been made-up as model martian soils, but it is believed that differences between the Amboy soil and later synthetic models would not be consequential to studies described here. The Amboy no. 6 soil sample was first mixed with 0.01% calcium superoxide to test sample handling and analytical techniques for the GEX simulation. The superoxide and soil were mixed to provide a suitable mass of sample for handling in the simulation apparatus, and the amount of superoxide alone was in the microgram range. The model soil had been ground, sieved, and remixed and its BET surface area was 22 m² g⁻¹. The oven-dried (423 K) sample was subjected to a humid atmosphere in the GEX simulation apparatus and released 1,820 nmol O₂, which was 5% more than calculated from reaction stoichiometry.

Because the sample handling and analytical techniques of the GEX simulation seemed adequate to test the release of small quantities of oxygen in a humid atmosphere, the release of oxygen from labile oxygen-containing surface complexes was tested next. A 1 g sample of Amboy no. 6 soil was pretreated to remove adsorbed water and equilibrated with a simulated martian atmosphere. The simulated martian atmosphere was

made and analysed by Matheson Gas, as 2.50 volume % N₂, 0.33 volume % O₂, 95.66 volume % CO₂, 1.35 volume % Ar, and 0.15 volume % CO. The moisture content of the mixture was 48 p.p.m. The Amboy no. 6 soil sample was subjected to a RF glow discharge in conditions chosen to expose the sample to active oxygen species at a high enough concentration level to accelerate the kinds of reactions possible between a soil and the naturally occurring active oxygen species at the surface of Mars⁹. A control sample was carried through the same time, temperature, and oxygen flow regime in the reactor, but without the RF field.

Figure 2 shows results obtained from the GEX humid mode simulation, and comparable results from VL-1 GEX humid mode data. The relative amounts of evolved gas species and their rates of evolution were similar for the GEX data and the simulation experiment, suggesting that the GEX oxygen release could have been the consequence of the long time of interaction of the martian soil with reactive oxygen species produced in the atmosphere by ultraviolet radiation.

The treatment of the VL-1 GEX humid mode data, together with the results of the simulation experiment, leads to several conclusions. First, the quantities of gases released at the temperature and humidity conditions of the GEX test cell were in agreement with a model of physical adsorption from the martian atmosphere on a soil of 17 m² g⁻¹ surface area, except for the anomalously large quantity of oxygen—which must be accounted for by oxygen adsorption in strongly bound or chemisorbed states, or as active oxygen compounds (for example, peroxide, superoxide, hydroperoxide) that decompose in a humid atmosphere.

Second, the results of the simulation of the GEX humid mode with RF glow discharge treated soil supports the possibility that the oxygen release seen in the GEX arose from labile oxygen surface species formed during long exposure of the martian soil to ultraviolet radiation induced active species in the atmosphere.

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E. VERNON BALLOU
PETER C. WOOD

Department of Chemistry,
San Jose State University,
San Jose, California 95192

THEODORE WYDEVEN
MARJORIE E. LEHWALT
RUTH E. MACK

Ames Research Center, NASA
Moffett Field, California 94035

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1. Klein, H. P. *et al. Science* **194**, 99–105 (196).
2. Oyama, V. I. *Icarus* **161** 167–184 (1972).
3. Oyama, V. I., Berdahl, B. J. & Carle, G. C. *Nature* **265**, 110–114 (1977).
4. Shorthill, R. W. *et al. Science* **194**, 91–97 (1976).
5. Owen, T. & Bieman, K. *Science* **193**, 801–803 (1976).
6. Braker, W. & Mossman, A. L. *Matheson Gas Data Book*, 5th edn (Matheson Gas Products, New Jersey, 1971).
7. Brown, F. S. *Viking Flight Team Memorandum to Biology Team*, Jet Propulsion Laboratory, Pasadena, 18 August 1976.
8. Robell, A. J., Borgardt, F. G. & Ballou, E. V. *Chem. Engng. Prog. Symp. Ser.* **62**, 76–80 (1966).
9. Barth, C. A. *Ber. Bunsenges. Phys. Chem.* **78**, 162–168 (1974); *A. Rev. Earth planet. Sci.* **2**, 333–367 (1974).

Energy basis of dielectric relaxation loss

JONSCHER has reviewed the phenomenology of dielectric, mechanical and some magnetic relaxations in a very wide range of materials¹. He found² that a fundamental insight into the physics of such processes may be obtained by considering that the ratio of the energy dissipated to the energy stored in these materials per radian of sinusoidal excitation is a constant which does not change over a very wide range of frequency.

This observation, the so-called 'universal dielectric response', has been derived by examining the behaviour of dielectric loss in the frequency and time domains, and tentatively modelled in terms of a partial screening mechanism²⁻⁴. Here we shall discuss Jonscher's work within the context of earlier work, and it will be shown that the presence of a loss peak in the frequency domain, or a change in the slope of the double logarithmic time-domain graph, need not require the two consecutive processes in the material that have been thought to be necessary^{1,4}. Subsequently, the clarified 'universal dielectric response' is used to interpret various empirical loci on the complex permittivity plane, Cole-Cole⁵, Davidson-Cole⁶ and Williams-Watts⁷⁻⁹ plots, and to compare these loci with the simple Debye form in terms of the behaviour of a single 'storage parameter', s , replacing Jonscher's n and m and the previously physically uninterpreted α of Cole and Cole and β of Davidson and Cole.

The classical Debye theory of dielectric relaxation¹⁰ involves the orientation of polar molecules in an applied field being resisted by the viscous damping of this rotation, as the molecules are considered as spheres in a continuous medium having the macroscopic viscosity of the material. On the complex relative permittivity plane

$$D^*(\omega)/\epsilon_0 E = \epsilon^*(\omega) - i\epsilon''(\omega) = 1 + \chi^*(\omega) \quad (1)$$

where ϵ_0 is the permittivity of free space, $8.845 \times 10^{-12} \text{ F m}^{-1}$, and $\chi^*(\omega) = \chi'(\omega) - i\chi''(\omega)$ is the susceptibility, the locus of ω predicted by the Debye theory is semicircular, with

$$\epsilon^*(\omega) - \epsilon_\infty = (\epsilon_s - \epsilon_\infty)/(1 + i\omega\tau) \\ = (\epsilon_s - \epsilon_\infty)/(1 + \omega^2\tau^2) - i(\epsilon_s - \epsilon_\infty)\omega\tau/(1 + \omega^2\tau^2) \quad (2)$$

In 1941 Cole and Cole⁵ noted that the dispersive mechanisms then used to explain dielectric relaxation loss, namely this Debye viscous damping or alternatively direct current conductivity, had the common characteristic of being wholly dissipative in nature, and that this was represented in the equivalent circuit of the dielectric by a pure resistance (Fig. 1a). However, they also showed that the behaviour of a very large number of dielectrics produced not a semicircle on $\epsilon^*(\omega)$, but a minor arc of a circle having its centre 'below' the real axis, described by

$$\epsilon^*(\omega) - \epsilon_\infty = \frac{(\epsilon_s - \epsilon_\infty)}{1 + (i\omega\tau)^{1-\alpha}} \quad (3)$$

and requiring that the dispersive mechanism producing the loss should not be represented by the purely dissipative Debye resistor, but rather by a complex 'polarisation impedance' $R(i\omega\tau)^{-\alpha}$, Fig. 1b. The loss peak produced in consequence by the Cole-Cole equivalent circuit is described by

$$\epsilon''(\omega) = \chi''(\omega) = \frac{(\epsilon_s - \epsilon_\infty) [\cos(\alpha\pi/2) (\omega\tau)^{1-\alpha}]}{1 + 2 \sin(\alpha\pi/2) (\omega\tau)^{1-\alpha} + (\omega\tau)^{2(1-\alpha)}} \quad (4)$$

which can be compared with the Debye term in equation (2). Both loss peaks are illustrated in Fig. 2.

In the words of Cole and Cole⁵, using the polarisation impedance "implies a conservation or 'storage' of energy in addition to dissipation of energy in the mechanism of molecular interaction responsible for the dispersion". As the vacuum response of the material is accounted for within the small parallel capacitive branch of the equivalent circuit, when

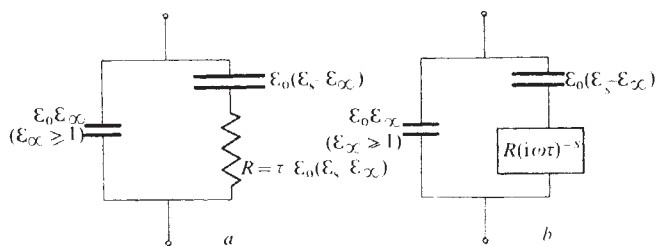


Fig. 1 Equivalent circuits of dielectric relaxation processes: a, Debye; b, Cole-Cole: s is a constant, α ; general: s may be a function of $(\omega\tau)$.

considering the properties of the boxed element in isolation we may write that the dispersive mechanism has

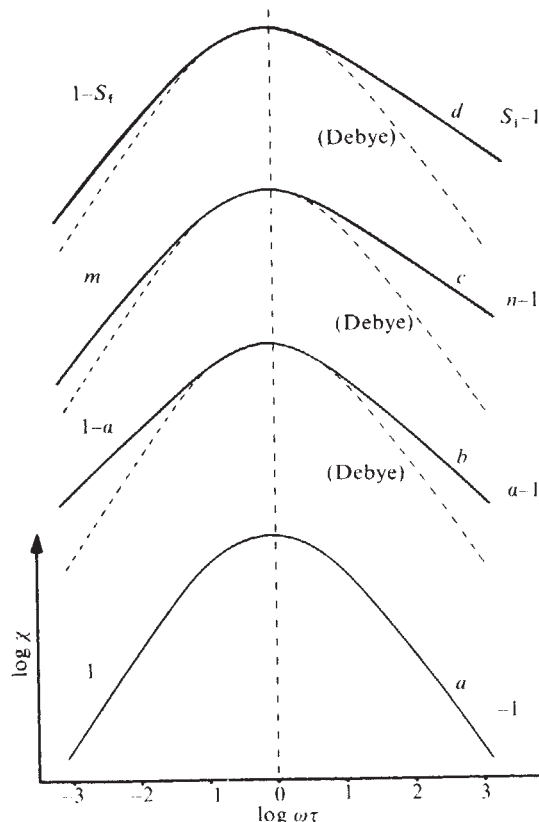
$$\epsilon_0 \epsilon'_b = \epsilon_0 \chi'_b = \epsilon_0 (\epsilon_s - \epsilon_\infty) \sin(s\pi/2) (\omega\tau)^{s-1} \quad (5)$$

$$\epsilon_0 \epsilon''_b = \epsilon_0 \chi''_b = \epsilon_0 (\epsilon_s - \epsilon_\infty) \cos(s\pi/2) (\omega\tau)^{s-1}$$

$$\text{and hence } \epsilon''_b/\epsilon'_b = \chi''_b/\chi'_b = \cotan(s\pi/2) \quad (6)$$

where s is a storage parameter equal in the Cole-Cole dielectric to a constant value, α . Consequently Cole and Cole⁵ concluded that "the ratio of the average energy stored to the average energy dissipated per cycle in the form of heat is a constant independent of frequency". This principle leads to a constant phase-angle, $(-s\pi/2)$ in the case of the polarisation impedance, as was first realised according to Cole¹¹ by Irving Wolff in the late 1920s. It also follows^{10,12} from the pioneering nineteenth century time-domain investigations of dielectric loss by Hopkinson, who produced evidence of power-law decay corresponding to parts of Fig. 3, as did Kohlrausch less completely in 1854.

Fig. 2 Frequency domain: the symbols show the slopes of the lines to which the loss peaks are asymptotic. The peaks are vertically separated for clarity. a, Debye; b, Cole-Cole; c, Jonscher; d, general.



Jonscher has discussed the principle in terms of Hilbert transforms¹³, calling it the 'universal dielectric response'. He has made^{2,3} the useful extension

$$\epsilon''_b/\epsilon'_b = \chi''_b/\chi'_b = (1-p)/p = \cotan(s\pi/2) \quad (7)$$

where p is the proportion of energy stored during a cycle of the field. However, the storage of a constant proportion of energy during each cycle of the field probably results from the storing of a constant proportion of energy during each of the operations of the dispersive mechanism that can take place before the field-reversal at the end of a half-cycle of the applied frequency. It is, therefore, more fundamental to consider each operation of the dispersive mechanism itself. If during this operation a proportion p of the energy is stored (Fig. 4), then equation (7) follows. Of course, if all the energy is dissipated, then the Cole-Cole α is zero, as the polarisation impedance reverts to the Debye resistor when p and s go to zero (cf. ref. 3). The Debye case (Fig. 2a) is the limit of zero energy storage within

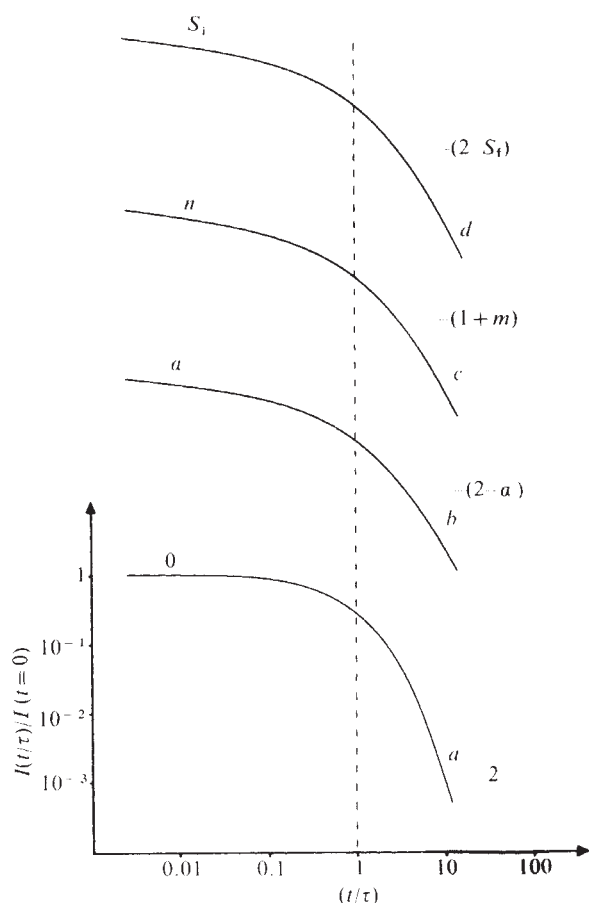


Fig. 3 Time domain: the symbols again show the slopes of the lines to which the curves are asymptotic. The non-Debye curves are vertically displaced for clarity. *a*, Debye; *b*, Cole-Cole; *c*, Jonscher; *d*, general.

the dispersive mechanism. However, unlike the situation in the viscous rotation of the Debye dipole sphere, in most real systems the necessary work will probably be used to separate the moving charge (for example, a pinned dipole^{2,3}) from its counter-charge atmosphere of partial screening, as described by polaron theory^{14,15}, and there will therefore also be some stored energy involved in the system due to the polaronic nature of the moving charge carrier. Although a theoretical investigation of the time course of the local amount of such stored energy might be somewhat difficult, the constant ratio of equation (7) follows

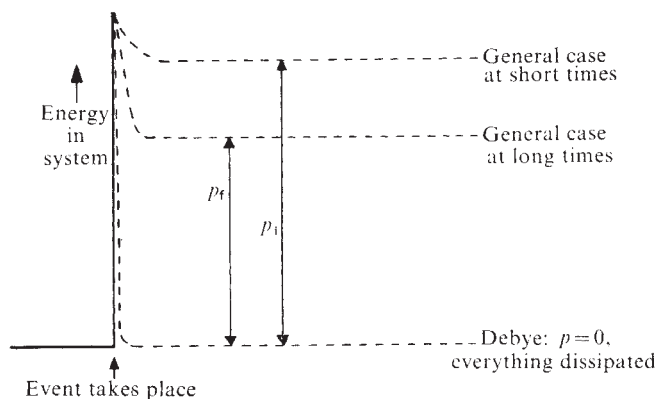


Fig. 4 Schematic diagram of the inferred progress with time of energy storage within an operation of the dispersive mechanism.

when there is inevitably involved, during each dipole jump and operation of the dispersive mechanism, a certain amount of energy storage associated with a certain amount of energy dissipation, and the field has, of course, to supply both. A change in the frequency of the field may affect the numbers jumping, but not the proportionality between the requisite dissipation and storage of energy related to these jumps. If no energy is stored and all is dissipated, we revert to the Debye case, Fig. 2a.

A single and unchanging proportion of storage p corresponding to a single value of s ($= \alpha$) can therefore lead to a definite (and broadened) loss peak, Fig. 2b, without involving two different and consecutive relaxation processes^{1,4}, which thus removes the only apparent enigma hindering the progress of a Jonscher-type interpretation of relaxation loss. The associated change in the exponent of the power-law loss with frequency, equation (4) and Fig. 2b, is both empirically necessary and theoretically acceptable: the Cole-Cole equation (3) has been shown⁵ to obey the Kramers-Kronig relations, modelling the entire necessary equivalent circuit (Fig. 1b) rather than just the polarisation impedance of the particular Hilbert transform demonstration of Jonscher¹³.

In the time domain Cole and Cole¹² further showed that to have not only a finite initial charge as elapsed time t tends to zero, but also a finite total charge transfer as t tends to infinity, the power-law decay of current resulting from step-potential stimulation has to have a knee (Fig. 3). As this occurs for a single value of energy storage p corresponding to a single value of s ($= \alpha$) across the time range, and applies also in the Debye case of $p = s = 0$, again there is no need to postulate two consecutive processes¹ as, of course, is required to agree with the frequency domain simplification, the loss peak in the frequency domain corresponding to the knee in the time domain.

The asymptotic slopes of the knee, however, are not always those to be expected from the Cole-Cole theory¹ (unless Jonscher's $(n-1)$ equals his m)³. This discrepancy is confirmed by much experimental data^{3,7-9}, and is extremely important as the inference is that p cannot be assumed to be constant in all cases across an arbitrarily wide range of time compared with the time constant τ of the relaxation mechanism. The initial proportion p_i (Fig. 4) and the 'final' proportion p_f correspond through equation (7) with values s_i and s_f of the storage parameter s that are manifested in the time domain as the general slopes of Fig. 3, and in the frequency domain as the asymmetrically broadened loss peak Fig. 2d. This concept will be elaborated quantitatively elsewhere¹⁶, and the required form of decay of p shown to be reasonable.

Meanwhile, let us briefly and qualitatively compare events in the time domain with their results on the permittivity plane, taking t as the reciprocal of the applied frequency $\omega/2\pi$ and assuming the absence of d.c. conductivity, which would mask the dielectric relaxation behaviour. Then if p decays to zero

extremely quickly, before even the time corresponding to t/τ very small ($\omega\tau$ very large), we find Debye results, a semicircle, on $\epsilon^*(\omega)$. If at least p decays to zero by the time corresponding to t/τ very large ($\omega\tau$ very small), then we find Davidson-Cole⁶ results on $\epsilon^*(\omega)$; the Davidson-Cole parameter β will be interpreted elsewhere¹⁶. But if p has not fully decayed to zero by this time corresponding to t/τ very large ($\omega\tau$ very small) we see something like Williams-Watts⁷⁻⁹ behaviour on $\epsilon^*(\omega)$, and if p does not decay at all from its initial value by the time that t/τ is very large, then Cole-Cole behaviour is seen on $\epsilon^*(\omega)$. Such effects can either be deduced intuitively by comparing Fig. 2 of ref. 12, with those given here, or derived analytically¹⁶. Thus combining the work of Cole and Cole with Jonscher's effective demonstration of the inconstancy of their parameter α , qualitatively explains all the experimentally observed types of dielectric relaxation. Quantitative results can be expected to advance with polaron theory.

D. C. SALTER

The Slade Hospital,
Headington,
Oxford, UK

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1. Jonscher, A. K. *Nature* **267**, 673-679 (1977).
2. Jonscher, A. K. *Nature* **253**, 717-719 (1975).
3. Jonscher, A. K. *Coll. Polym. Sci.* **253**, 231-250 (1975).
4. Jonscher, A. K. *Nature* **256**, 566-568 (1975).
5. Cole, K. S. & Cole, R. H. *J. chem. Phys.* **9**, 341-351 (1941).
6. Davidson, D. W. & Cole, R. H. *J. chem. Phys.* **19**, 1484-1490 (1951).
7. Williams, G. & Watts, D. C. *Trans. Faraday Soc.* **66**, 80-85 (1970).
8. Williams, G., Watts, D. C., Dev, S. B. & North, A. M. *Trans. Faraday Soc.* **67**, 1323-1335 (1971).
9. Johari, G. P. *Ann. N. Y. Acad. Sci.* **279**, 117-140 (1976).
10. Daniel, V. V. *Dielectric Relaxation* (Academic, London, 1967).
11. Cole, K. S. *Membranes, Ions and Impulses* (University of California, Berkeley, 1972).
12. Cole, K. S. & Cole, R. H. *J. chem. Phys.* **10**, 98-105 (1942).
13. Jonscher, A. K. *Nature* **250**, 191-193 (1974).
14. Bosman, A. J. & van Daal, H. J. *Adv. Phys.* **19**, 1-117 (1970).
15. Mott, Sir Nevill & Davis, E. A. *Electronic Processes in Non-Crystalline Materials* (Clarendon, Oxford, 1971).
16. Salter, D. C. *Proc. R. Soc. Lond. A* (to be submitted).

Pressure retardation of vitrinite diagenesis, offshore north-west Europe

PHYSICO-CHEMICAL processes that increase coal rank also increase vitrinite reflectance, or the level of vitrinite diagenesis. The principal physical factors controlling these processes have been identified as geothermal gradient, thermal conductivity of lithological section, duration of heating, and pressure^{1,2}. Interaction of these factors on coalification has not been demonstrated conclusively in nature or by laboratory experiments, but temperature and time have been generally regarded³ as most influential. Although the retarding effect of pressure has been widely recognised, the role of pressure is not clearly understood and has been considered³ negligible. Here the same values of mean vitrinite reflectance in oil ($R_{mo}\%$) have been related to present temperature, (T), and to a function of the present temperature and the ratio of the gradients of normal hydrostatic pressure (P_{NH}) and observed pore pressure (P_p), $T(\gamma P_{NH}/\gamma P_p)$, in Fig. 1a and b respectively. Comparison of correlation coefficients of Fig. 1a and b demonstrates that, for values of R_{mo} less than 1.50%, retardation of vitrinite diagenesis by abnormally high formation pressure, is both significant and predictable. As a corollary, the time factor seems to be of limited significance on a geological scale.

For Fig. 1a and b, values of vitrinite reflectance have been restricted to those from coals and non-calcareous shales from several wells offshore from north-west Europe. Sections sampled in these wells do not show evidence of uplift. The samples range in age from mid-Tertiary to Carboniferous. The wide range of ages might not be considered ideal. It is more important, however that present depths of burial and present temperatures are likely to be the maximum suffered by the samples.

The three types of pressure distinguished in geological sections, pore pressure (P_p), effective overburden pressure

(P_{EO}), and geostatic pressure (P_G), are related according to the simple formula

$$P_G = P_p + P_{EO} \quad (1)$$

Following Gretener⁴, geostatic pressure gradient (γP_G) is taken to be 1.00 psi ft⁻¹ (= 0.231 kg cm⁻² m⁻¹). In offshore north-west Europe, the pore pressure gradient of normally pressured sequences, normal hydrostatic pressure, (γP_{NH}) is generally about 0.45 psi ft⁻¹, and gradient of effective overburden pressure (γP_{EO}) is, therefore, 0.55 psi ft⁻¹. Some samples for vitrinite reflectance have come from normally pressured sequences but others are from abnormal, overpressured intervals. In these intervals, γP_p exceeds γP_{NH} , and is generally greater than 0.50 psi ft⁻¹. Conversely, γP_{EO} in the overpressured intervals is less than 0.50 psi ft⁻¹.

Many techniques for estimating formation pressures are available^{5,6}. Formation tests yield the best pressure information, but not enough have been conducted in the wells studied for composition of a satisfactory pressure profile. Therefore, profiles of sonic transit times in shales (Δt_s) penetrated in the wells have been favoured in this study for estimation of formation pressures⁶⁻⁸. Estimates of pressure by means of this technique, provide sufficient values of pressure for plotting pressure profiles of high precision in individual wells.

Values of present temperature (T), in °F, were estimated by interpolation between control temperatures. Horner-type plots of log recorded, bottom hole temperatures⁹ were used to determine control temperatures. During normal compaction of porous, water saturated shales, geostatic pressure increases as depth of burial increases. Decrease in shale porosity and consequent reduction in pore water content relate to increase in geostatic pressure and effective overburden pressure^{10,11}.

Figure 2, based on data from Lewis and Rose¹¹, shows that the coefficient of thermal conductivity of water saturated, porous shale increases with decrease in water content according to the formula:

$$K_t = (K_s K_r \phi / K_s) \quad (2)$$

where K_t is thermal conductivity of total shale; K_s is thermal conductivity of solid shale (= 1.138 Btu h⁻¹ ft⁻¹ °F⁻¹), K_r is thermal conductivity of fluid (water = 0.363 Btu h⁻¹ ft⁻¹ °F⁻¹), and ϕ is shale porosity (where 1 Btu h⁻¹ ft⁻¹ °F⁻¹ = 1.488 kg cal h⁻¹ m⁻¹ °C⁻¹). It is important that thermal conductivity of water does not change significantly with variations in salinity⁶.

In overpressured section, shale porosity (ϕ), effective overburden pressure, and pore-water content are the same as at shallower depths in normally compacted shale sequences. Thus thermal conductivity will be abnormally low in overpressured sequences, whatever the depth, pressures, and porosity of the overpressured shale-water system.

Reduction in thermal conductivity of overpressured shales causes increase in thermal gradient of the overpressured sequence, according to the equation for heat flow¹¹

$$Q = (k_t/L) a \Delta T \quad (3)$$

where k_t is thermal conductivity of shale section, 'L' is thickness of shale section, and ΔT is temperature change along section of thickness 'L', that is the thermal gradient.

Overpressured shale is a heat insulator and normally pressured shale is a heat conductor¹¹. In a state of thermal equilibrium, however, heat flow in an overpressured sequence (Q_{op}) equals that in normally pressured sequence (Q_{np}) of the same section. For this condition the equation for thermal gradient of the overpressured section (ΔT_{op}) is

$$\Delta T_{op} = (\Delta T_{np} (k_{np}/k_{op})) \quad (4)$$

Therefore, the thermal gradient in the overpressured section will be greater than that of the normally pressured section.

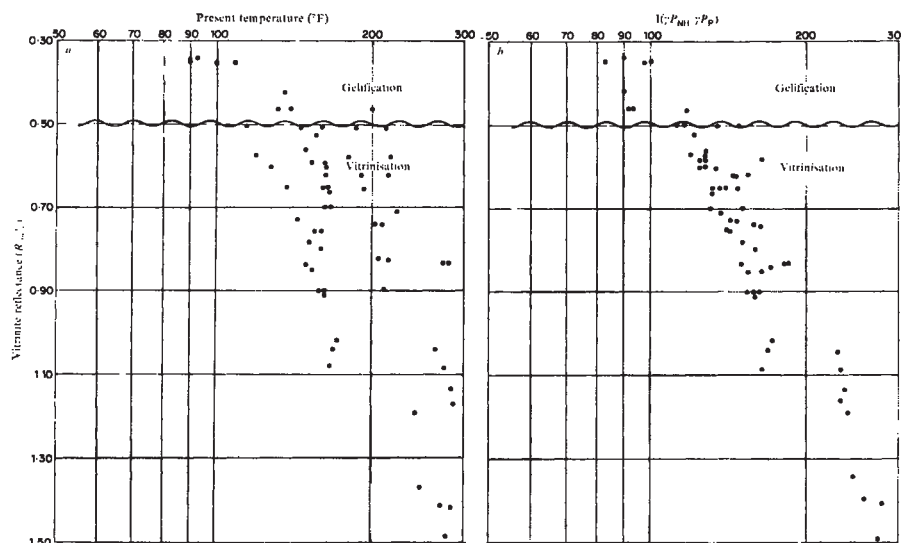


Fig. 1 a, Relationship of vitrinite reflectance ($R_{mo}\%$) to present temperature (T). Temperature in $^{\circ}\text{F}$. Correlation coefficient (r) is 0.716 for the equation $\log_n T = 1.998 + 0.326 R_{mo}\%$. b, Relationship of vitrinite reflectance ($R_{mo}\%$) to the pressure-temperature function ($T(\gamma P_{NH}/\gamma P_P)$). Correlation coefficient (r) is 0.952 for the equation $\log_n T(\gamma P_{NH}/\gamma P_P) = 1.838 + 0.433 R_{mo}\%$.

In a subsurface section, the temperature (T_d) at a given depth (D) is determined from the equation

$$T_d = T_s + D \Delta T \quad (5)$$

where T_s is the mean surface temperature. Hence, the temperature at a given depth in an overpressured interval will be greater than the temperature at the same depth in a normally pressured interval for the same type of lithology. Conversely, for a given temperature, heat flow will be lower in an overpressured interval than a normally pressured interval.

The low correlation coefficient ($r = 0.716$) of the relationship of $R_{mo}\%$ to present temperature (T) of Fig. 1a contrasts with the high order of correlation ($r = 0.952$) of the relationship $R_{mo}\%$ to $T(\gamma P_{NH}/\gamma P_P)$ of Fig. 1b. The improved correlation coefficient for the function $T(\gamma P_{NH}/\gamma P_P)$ demonstrates that the influence of abnormal formation pressure must be considered when evaluating the relationship of vitrinite reflectance to natural, subsurface physical conditions.

Coalification has been regarded as a first-order reaction³; the same is probably true of vitrinitisation. In this case, improvement in correlation noted above can be explained readily by reference to the Arrhenius equation

$$k = A \exp(-E/RT) \quad (6)$$

where k is the reaction rate; A is the Arrhenius factor; and $\exp(-E/RT)$ is the Boltzmann factor (= fraction of molecules with energy equal to or greater than the critical activation energy E). From this equation

$$\log_n k = -E/RT + \log_n A \quad (7)$$

where $\log_n A$ is a constant.

By substituting in equation (7) for temperatures T_n (normally pressured) and T_o (overpressured), reaction rates k_n and k_o , at temperatures T_n and T_o , respectively, can be compared according to the formula

$$\log_n (k_o/k_n) = \frac{E}{R} \left(\frac{1}{T_n} - \frac{1}{T_o} \right) \quad (8)$$

where temperatures are expressed in K.

For coeval and identical values of $R_{mo}\%$, one at normal pressure, the other in an overpressured sequence, the tempera-

ture will be higher in the overpressured sequence and (k_o/k_n) will equal 1. However, values of T_n ($\gamma P_{NH}/\gamma P_P$) and T_o ($\gamma P_{NH}/\gamma P_P$) will be the same where T_n and T_o are expressed in $^{\circ}\text{F}$ or in K. This is because ($\gamma P_{NH}/\gamma P_P$) = 1 in a normally pressured sequence and ($\gamma P_{NH}/\gamma P_P$) < 1 in an overpressured sequence. Therefore from equation 8:

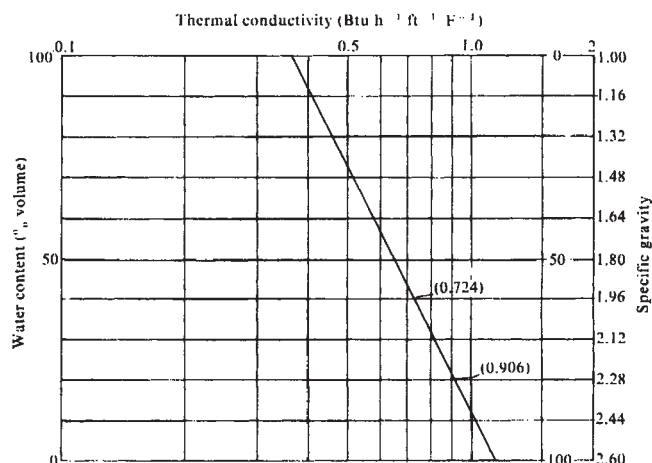
$$\log_n (1.0) = \frac{E}{R} \left(\frac{1}{T_n} \cdot \frac{\gamma P_{PN}}{\gamma P_{NH}} - \frac{1}{T_n} \cdot \frac{\gamma P_{PN}}{\gamma P_{NH}} \right) = 0$$

here $T(\gamma P_{NH}/\gamma P_P)$ should be expressed in K.

In Fig. 1b the scatter of points in the gelification stage might indicate a change in the gradient of increase of $R_{mo}\%$. But there are too few points to establish that the rate of increase in $R_{mo}\%$ in the gelification stage is different to that of the vitrinitisation stage, even though physico-chemical processes are different between these stages. In the gelification stage the role of pressure is probably greater than it is in vitrinitisation.

Detailed investigation of the relationship of effective overburden pressure to vitrinite reflectance is still in progress, but

Fig. 2 Relationship of shale water content to thermal conductivity of shale. Increase in thermal conductivity of shale is exponentially related to decrease in water content. Thermal conductivities of 0.724 and 0.905 $\text{Btu h}^{-1} \text{ft}^{-1} \text{ } ^{\circ}\text{F}^{-1}$ relate respectively to shale water contents of 40% and 20%.



preliminary results indicate a closer correlation to $R_{mo}\%$ than that of $T(\gamma P_{NH}/\gamma P_P)$. This may be due to more accurate measure of heating as P_{EO} measures confining pressure, reproducible in closed system laboratory studies. Effective overburden pressure can be determined at the levels of the sample taken for measurement of vitrinite reflectance.

The high coefficient of correlation of Fig. 1b has demonstrated that the combined effect of heat and pressure, especially overpressure, influences increase in vitrinite reflectance for values of R_{mo} less than 1.50%. Pressure has a determinative role in this combination, because of its influence on thermal conductivity of the geological section containing the vitrinite.

Figure 2 and equation (3) indicate that the thermal conductivity of shale in a thick continuous shale section will be the same for a given effective overburden pressure irrespective of its depth. This will be true for any area of uniform heat flow and will be the same whether the shale is overpressured or not.

Heat and time have been related to coalification and increase in vitrinite reflectance. In Fig. 1a and b the values of vitrinite reflectance are for the same samples, so the time-factor does not have to be considered in this comparison. Nevertheless, the comparison has important implications for the significance of time in coalification processes. The amount of time available for heating may influence the final state of maturation of vitrinite, coal or other hydrocarbons. From the excellent correlation of Fig. 1b it can be inferred that the time-factor in relation to precision of vitrinite reflectance estimates for given temperatures and pressures, is of limited significance on a geological scale and is likely to be critical only for a short period.

Further investigation of the combined effect of heat, pressure, thermal conductivity of sediment, and time in natural, geological conditions will improve understanding of the increase in reflectance of vitrinite. The resulting data should also provide a more sound basis for interpretation of the role of natural physical processes in the maturation of all types of organic matter in terms of physical chemistry.

R. A. McTAVISH

Continental Oil Company Ltd,
116 Park Street,
London W1, UK

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1. Teichmüller, M. & Teichmüller, R. in *Coal and Coal-bearing Strata* (eds Murchison, D. G. & Westcott, T. S.) 233–267 (Oliver and Boyd, Edinburgh, 1968).
2. Ammosov, I. I. *C.R. 6ème Congres Int. Strat. Geol. Carbonifere* 2, 403–415 (1970).
3. Lopatin, N. V. & Bostick, N. H. *Reprint III, Geol. Surv. 1974–Q*, 1–16 (1974).
4. Gretener, P. E. *Bull. Can. petrol. Geol.* 17, 255–259 (1969).
5. Timko, D. J., Fons, L. C., Gritman, W. K. & Rees, F. B. in *Abnormal Sub-surface Pressure, A Study Group Report 1969–1971*, 20–30 (Houston Geological Society, Houston, 1971).
6. Fertl, W. H. *Abnormal Formation Pressures* (Elsevier, Amsterdam, 1976).
7. Hottman, C. E. & Johnson, R. K. *J. petrol. Technol.* 17, 717–723 (1965).
8. Wichmann, P. A. *Trans. III Europ. Form. Eval. Symp.* S1–S15 (1974).
9. Dowdle, W. L. & Cobb, W. M. *J. petrol. Technol.* 27, 1326–1330 (1975).
10. Jones, P. H. *J. petrol. Technol.* 21, 803–810 (1969).
11. Lewis, C. R. & Rose, S. C. *J. petrol. Technol.* 22, 11–16 (1970).

Rejecting evidence of Gothenberg geomagnetic reversal in New Zealand

MORNER and Lanser¹, in 1974, gave evidence that the Gothenberg Geomagnetic Event had been discovered in New Zealand. The report was made prematurely, being based on remarks by Topping² in an address at the INQUA Congress in Christchurch, New Zealand, 1973. Further investigations, detailed here, show that the presumed evidence for the Gothenberg reversal was unsound.

Topping's presumption was based on measurements of palaeomagnetic polarities in 16 andesite boulders taken from two adjacent localities in a lahar. The lahar is between 9,540- and 12,450-yr-old (ref. 2) and is located at Bruce Road on the northwestern side of Mount Ruapehu, National Park, New Zealand. Eleven of the boulders displayed

reversed polarities, and Topping concluded that this was sufficient to prove that the lahar was hot when it flowed, and the boulders had not cooled to below their Curie temperatures until after the lahar had come to rest. The independently determined age of the lahar, based primarily on ¹⁴C measurements³, indicated correlation of the implied reversed geomagnetic field with the Gothenberg Event. If correct, this would have been the first record of the Gothenberg Event in the Southern Hemisphere, and in view of its importance more thorough investigation was obviously needed.

Therefore an additional 16 oriented boulders of andesite were taken from one of Topping's localities. Two, three or four samples, at different distances between the outer surface and centre, were taken from each boulder, and polarities were measured with a spinner magnetometer.

Only a few samples had been magnetically cleaned by Topping. They showed no sign of instability after thermal demagnetisation at 150 °C, and Topping assumed that the magnetisations of all his sample boulders were stable. The following more rigorous cleaning tests confirm that Topping's assumption was correct.

Samples from three of the newly collected boulders were thermally demagnetised step by step at 50° intervals between 100 °C and 600 °C, and samples from two others were a.c. demagnetised at peak field intervals of 10 mT up to 50 mT. Directional changes were negligible up to 500 °C for thermal demagnetisation and up to 50 mT for a.c. demagnetisation. The palaeomagnetic stability index of Briden³ was applied and confirmed these observations. From these stability indices a peak field of 10 mT was chosen as best for a.c. cleaning and a maximum temperature of 300 °C for thermal cleaning. The remaining samples were cleaned at either of the above values, approximately half by each method.

The magnetisation directions of samples from each individual boulder are in good agreement with one another. This shows that the material in each boulder was at rest while cooling from the Curie temperature.

The magnetisation directions of individual boulders seem to be random, showing that their natural remanent magnetisation (NRM) could not have been acquired by cooling after coming to rest in the lahar mound.

After cleaning, the mean inclination for all the boulders was -10° with a standard deviation of 35° , and the mean declination was 132° with a standard deviation of 72° . A *t*-test shows that the inclinations and declinations are randomly distributed at the 95% confidence level.

It is thus concluded that the volcanic material composing the boulders acquired its thermoremanent magnetisation (TRM) by cooling in a stable magnetic field before the boulders were involved in the lahar flow. During the lahar flow the rocks were below their Curie temperature, and when they came to rest were magnetically oriented in random directions. They therefore do not record a geomagnetic field direction and are not suitable for testing the existence in the Southern Hemisphere of the Gothenberg Reversed Event.

J. C. SUKROO
D. A. CHRISTOFFEL

Physics Department,
Victoria University of Wellington,

P. VELLA
W. W. TOPPING

Geology Department,
Victoria University of Wellington,
Wellington, New Zealand

Received 10 October; accepted 6 December 1977.

1. Morner, N. A. & Lanser, J. L. *Nature* 251, 408 (1974).
2. Topping, W. W. thesis, Victoria Univ. Wellington (1974).
3. Briden, J. C. *J. geophys. Res.* 77, 1401 (1972).

Non-biogenic fixed nitrogen in Antarctica and some ecological implications

TOTAL fluxes of nutrients in ecosystems and the biosphere have been estimated¹⁻³, especially for tropical and midlatitude regions, but no study has attempted to estimate the contribution of fixed nitrogen from the Antarctic ice sheet. Using data from analyses of South Pole ice, we estimate here that the mean annual accumulation of fixed nitrogen in the Antarctic ice sheet is 2.73×10^4 tonnes of nitrate (as N) and 1.88×10^4 tonnes of ammonium (as N). If annual snow accumulation approximates annual losses through calving of icebergs, blowing snow and melting of the bottom of ice shelves, then Antarctic Ocean surface waters will be enriched by 4.61×10^4 tonnes of $\text{NO}_3\text{-N} + \text{NH}_4\text{-N}$ during the austral summer. This contribution of fixed N to circumpolar surface waters may significantly influence productivity.

A total of 364 South Pole ice core samples from various depths were analysed for ammonium ion (phenol-hypochlorite method⁴), nitrate ion (diazotisation method⁵), and nitrate ion (cadmium reduction method⁵). These methods do not detect other reduced NH_x species which might be present in trace amounts. Details of corehandling and analytical methods are reported in Parker *et al.*⁶.

Table 1 summarises our results. Almost no $\text{NO}_2\text{-N}$ was found except in the upper few metres of snow contaminated by fuel combustion products⁶. Average annual accumulation rate estimates at the South Pole range over 6–10 cm of water⁷⁻¹⁰. Based on estimates of annual accumulation rates at various points, an average value of 10 cm of water per year for the entire ice sheet seems reasonable¹⁰. The continent measures $14\text{--}15 \times 10^6$ km² and is > 95% ice-covered with an estimated mean ice thickness of 2.0 km. Using 14×10^6 km² as the ice sheet area, total glacial ice volume is about 28×10^6 km³ with a mean density of nearly 1.0. The assumption that our measured nitrogen values are representative of the entire mass of ice is probably conservative, because coastal Antarctica receives additional nitrogen input from sources such as birds and coastal nitrogen-fixing biota. We calculate the following, assuming a mean density of 1.0 for glacial ice:

- (1) Mean annual $\text{NO}_3\text{-N}$ input to the continent,
 $(14 \times 10^6 \text{ km}^2) \times (10^6 \text{ m}^2 \text{ km}^{-2}) \times (19.5 \mu\text{g NO}_3 \text{ N l}^{-1}) =$
 $2.73 \times 10^{10} \text{ g} = 27,300 \text{ tonnes} (= 121,000 \text{ tonnes of NO}_3^-)$.
- (2) Mean annual $\text{NH}_4\text{-N}$ input to the continent,
 $(14 \times 10^6 \text{ km}^2) \times (10^6 \text{ m}^2 \text{ km}^{-2}) \times (13.4 \mu\text{g NH}_4 \text{ N l}^{-1}) =$
 $18,800 \text{ tonnes} (= 24,100 \text{ tonnes of NH}_4^+)$.
- (3) Mean annual $\text{NO}_3\text{-N} + \text{NH}_4\text{-N}$ input to the continent,
 $(1) + (2) = 46,100 \text{ tonnes} (= 145,100 \text{ tonnes of NO}_3^- + \text{NH}_4^+)$.
- (4) Total $\text{NO}_3\text{-N} + \text{NH}_4\text{-N}$ contained in the Antarctic ice sheet,
 $922 \times 10^6 \text{ tonnes} (= 2.902 \times 10^9 \text{ tonnes of NO}_3^- + \text{NH}_4^+)$.

This total fixed nitrogen in the Antarctic ice sheet is nearly five times Delwiche's³ estimate of annual world production from all sources.

Wilson and House¹¹, based on pooled samples of South Pole snow, calculated an annual infall of N (as NO_3^- plus NO_2^-) of 0.0045 lb per acre ($= 7.06 \times 10^6$ kg per year over the continent). Our calculations, which include $\text{NH}_4\text{-N}$, indicate an average of 27.3×10^6 kg or nearly four times their estimate. Wilson and House¹¹ found no NH_4^+ , but they used the direct

Nezzlerisation method, which does not detect NH_4^+ below about $15 \mu\text{g N l}^{-1}$. We agree with Wilson and House that one probable mechanism for nitrogen fixation at the South Pole involves auroral activity, but we further propose that other stratospheric ionisation processes may add to the total fixed nitrogen in polar regions.

Our calculated $\text{NO}_3\text{-N}$ and $\text{NH}_4\text{-N}$ annual input of 46,100 tonnes to the Antarctic ice sheet is only about 0.37% of Delwiche's³ estimate of 7.4 M tonnes per year for the world's atmospheric N fixation. The nitrate- and ammonia-N in Antarctic ice is probably from natural atmospheric sources, a view strengthened by the fact that our analytical data include > 50 m of the South Pole firn core covering over 400 yr (Parker, unpublished). If the primary mechanism of nitrogen fixation is related to stratospheric ionisation over Antarctica, one must expect that a similar condition prevails in the Arctic, although tropospheric mixing patterns will differ in the two regions. Assuming atmospheric N fixation in both polar regions to be approximately equal, then about 0.74% of Delwiche's³ total is produced in polar regions between lat 70° and 90° and > 99% is produced between lat 70°N and 70°S; this latter zone includes about 80% of the earth's surface and has appreciably less auroral activity. Also, atmospheric nitrogen fixation by lightning^{12,13} and by photochemical oxidation¹³ is considered to be extremely small. Consequently Delwiche's estimate of 7.4 M tonnes per year for world atmospheric $\text{NO}_3\text{-N}$ production may be excessive. Delwiche³ has emphasised that appreciable uncertainty may result because of difficulties in correctly estimating the proportions, in rainfall, of fixed nitrogen derived from atmospheric ionisation and from land or sea. Our data largely reflect inputs from purely atmospheric processes, because the South Pole is remote from land, sea, and global human activities, also because much of the ice core examined predates the advent of massive industrialisation, and biota such as nitrogen-fixing organisms are absent at the South Pole.

The contribution by the Antarctic ice sheet to the nitrogen balance of the Southern Ocean seems to be significant. The ice sheet is apparently in volumetric equilibrium. Approximately 1.4×10^6 M tonnes per year of N-laden Antarctic ice, therefore, enters Antarctic circumpolar waters either as icebergs or from blowing snow and melting of the bottom of ice shelves. The icebergs contain $19.5 \mu\text{g NO}_3\text{-N}$ and $13.4 \mu\text{g NH}_4\text{-N l}$. Using the sum of these mean values, the annual contribution of inorganic nitrogen to the Southern Ocean is 4.61×10^4 tonnes.

Nitrate -N values for Antarctic circumpolar surface waters vary from 0.02 to $21.4 \mu\text{g l}^{-1}$, with a mean value of 2.7 (refs 14, 15). Icebergs should contain about 7.2 times this concentration of nitrate. Little data on $\text{NH}_4\text{-N}$ for the Southern Ocean is available. Icebergs are not randomly distributed in Antarctic circumpolar waters, but follow relatively discrete routes dictated by surface currents¹⁶. As icebergs melt, the fresh, low-density water, enriched in nitrogen, will tend to rise and remain at the surface and within the euphotic zone. Nitrogen is recognised to be a nutrient limiting to productivity in some oceanic regions, but is not generally so in Antarctica where higher inorganic nitrogen concentrations occur relative to other seas^{14,15}. We suggest that N-laden ice originating on the Antarctic continent provides a major contribution to the nitrogen budget of the epipelagic zone of the Southern Ocean.

The area of the Antarctic Circumpolar Ocean, between the continental margin and the Subarctic Convergence, is about 14×10^6 km² (ref. 17). The depth of the mixed layer approximates 50 m, so the volume of the Southern Ocean into which the Antarctic nitrogen is injected is about $50 \text{ m} \times 14 \times 10^{12} \text{ m}^2 = 7 \times 10^{14} \text{ m}^3 = 7 \times 10^{17} \text{ l}$. Using the estimate of mean $\text{NO}_3\text{-N}$ concentration ($2.7 \mu\text{g l}^{-1}$) of Balech *et al.*¹⁴, the mixed layer reservoir of $\text{NO}_3\text{-N}$ amounts to $(2.7 \mu\text{g l}^{-1}) \times (7 \times 10^{17} \text{ l}) = 19 \times 10^{11} \text{ g}$ or 19×10^5 tonnes of inorganic N (neglecting NO_2^- and NH_4^+). We have calculated above that the average annual input of $\text{NO}_3\text{-N}$ from Antarctica is 2.73×10^4 tonnes, which is 7% of the total in the reservoir.

In spite of the general agreement that phytoplankton pro-

Table 1 Means, standard errors of the mean, and range of values for the two species of fixed nitrogen

Species	$\bar{x}$	s.e. $\bar{x}$	Range
$\text{NO}_3\text{-N}$	19.57	14.34	0–69
$\text{NH}_4\text{-N}$	13.34	22.46	0–260

All values are in $\mu\text{g l}^{-1}$

ductivity is light (not nutrient) limited in the Antarctic, this input may have an effect completely out of proportion to its relative size because it is released into the euphotic zone at the time and place most beneficial to phytoplankton (that is, at the surface, as light begins to increase, both promoting photosynthesis and causing the icebergs to melt more quickly).

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Note added in proof: We have just confirmed that icebergs have concentrations near our estimates and that a concentration gradient occurs, descending with increasing distance from an iceberg.

BRUCE C. PARKER
LAWRENCE E. HEISKELL
WILLIAM J. THOMPSON

Biology Department, Virginia Polytechnic Institute & State University, Blacksburg, Virginia 24061

EDWARD J. ZELLER

Department of Geology, University of Kansas, Lawrence, Kansas 66045

Received 27 September 1977; accepted 3 January 1978.

1. Hornbeck, J. W. & Likens, G. E. in *Advanced Concepts and Techniques in the Study of Snow and Ice Resources* 139–151 (N.A.S., Washington, D.C., 1974).
2. Kormondy, L. J. *Concepts of Ecology* (Prentice-Hall, Englewood Cliffs, New Jersey, 1976).
3. Delwiche, C. C. *AMBIO* 6, 106–111 (1977).
4. Martin, D. F. in *Marine Chemistry*, I, 143–158 (Marcel Dekker, New York, 1972).
5. American Public Health Assoc. *Standard Methods for the Examination of Water and Wastewater* 13th edn, 453–534 (1971).
6. Parker, B. C., Zeller, E. J., Heiskell, L. E. & Thompson, W. J. *Antarctic J. U.S.* (in press).
7. Gow, A. J. *J. Glaciol.* 5, 467–477 (1965).
8. Epstein, S., Sharp, R. P. & Gow, A. J. *J. geophys. Res.* 70, 1809–1814 (1965).
9. Giovinetto, M. B. *Trans. geophys. Union* 42, 386–389 (1961).
10. Bull, C. in *Research in Antarctica* 367–421 (AAAS Publ. no. 93, Washington, D.C., 1971).
11. Wilson, A. T. & House, D. A. *Nature* 205, 793–794 (1965).
12. Noxon, J. F. *Geophys. Res. Lett.* 3, 463–465 (1976).
13. Soderlund, R. *AMBIO* 6, 119–122 (1977).
14. Balech, E., El-Sayed, S. Z., Hasle, G., Neushul, M. & Zaneveld, J. S. in *Antarctic Map Folio Series, Folio 10* (American Geographical Society, 1968).
15. Spencer, C. P. in *Chemical Oceanography* 2nd edn, 2, 277 (Academic, New York, 1975).
16. Swinbank, C., McClain, P. & Little, P. *Polar Rec.* 18, 495–501 (1977).
17. Sverdrup, H., Fleming, R. & Johnson, M. *Oceans: Their Physics, Chemistry, and General Biology* (Prentice-Hall, New York, 1942).

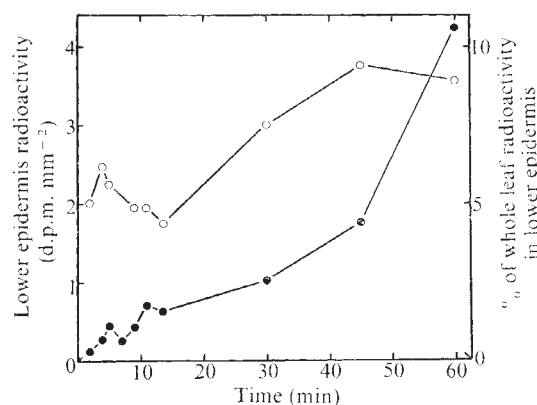


Fig. 1 Uptake of radioactivity by the lower epidermis of *Commelina* leaves fed $2\text{-}^{14}\text{C}$ -ABA through the transpiration stream. Plants of *C. communis* were grown from seed and used when about 7 weeks old. Two-leaved shoots from non-flowering plants were pretreated to obtain open stomata by floating on water through which CO_2 -reduced air was bubbled. After drying, they were stood in vials containing $2\text{-}^{14}\text{C}$ -ABA solution ($3\text{ }\mu\text{Ci ml}^{-1}$, 11.7 Ci mol^{-1}) 250 mm from two 60-W incandescent bulbs at ambient temperatures. Shoots were collected at various times and 50-mm² square abaxial epidermis samples were taken for radioassay (●) and autoradiography. The radioactivity present in the corresponding mesophyll plus adaxial epidermis square was also estimated and used to calculate the percentage of whole leaf radioactivity present in the lower epidermis (○). Radioassay was by liquid scintillation spectrometry after extraction for 3 h in 1 ml methanol:ether (1:1, v:v) mixture. Allowance was made for the quenching caused by the leaf material. Each point on the graph is the mean of three samples from one leaf. Estimates from 20 measurements of stomatal aperture from freeze-dried material indicated that closure was complete after 9 min of $2\text{-}^{14}\text{C}$ -ABA uptake.

for the amount of ABA required to cause stomatal closure. Using an estimate of 50 stomata per mm², we calculate that the stomata had closed after receiving no more than 300 amol of ABA per stomatal complex. Because at the time of closure 5% of the total radioactivity was present in the lower epidermis, this represents 300 fmol per mm² leaf area. This figure confirms the data of Raschke¹¹ who calculated that 90–180 fmol of ABA per mm² leaf area was required to give 5% stomatal closure in *Commelina*. Kriedemann *et al.*¹⁴ estimated that 89–349 fmol of ABA per mm² leaf area was required to initiate closure in French bean, rose and *Zea* leaves.

Microautoradiography (Fig. 2a, b) reveals a distinct correlation between the aggregation of silver grains in the film emulsion and the distribution of stomatal complexes in the tissue. This is not restricted to the pattern of $2\text{-}^{14}\text{C}$ -ABA uptake in isolated epidermes, because similar distributions of silver grains were obtained in autoradiograms from epidermes in which the label was applied through the transpiration stream for 1 h. Evidence from thin-layer chromatography suggests that more than 90% of the radioactivity present in methanol: ether extracts of epidermal tissue incubated in $2\text{-}^{14}\text{C}$ -ABA for 1 h was chromatographically identical to ABA. The mobility of the labelled molecules was manifest in the fact that more than 80% of the radioactivity initially present in such tissue had been lost to the efflux medium after 90 min of incubation in 1 mM CaCl_2 solution. This indicated that distribution studies using ABA should be restricted to the techniques of soluble-compound autoradiography. Neither the net uptake nor the distribution pattern of radioactivity in isolated strips incubated for 1 h was significantly affected by pretreatments causing opening or closing of the stomata.

We have shown that radioactivity from labelled ABA becomes localised in the region of the guard cells of *Commelina communis* after two different treatments. It may be argued that the distribution found is commensurate with that expected of a hormone reaching its target cells. Nevertheless, several points need further clarification, particularly the following. (1) The time courses used for the autoradiography were longer than those required to cause stomatal closure; shorter presentation

Abscisic acid and guard cells of *Commelina communis* L.

THE plant hormone abscisic acid (ABA) is thought to be involved in the regulation of transpiration through its effects on stomatal aperture¹. Application of synthetic ABA causes stomatal closure in whole plants², shoots³ and leaf epidermal strips⁴. During water stress, levels of the hormone increase in plant tissues⁵, including the leaf epidermis⁶, and this is usually associated with stomatal closure⁷. There is evidence that ABA affects the ionic^{8,9} and metabolic⁹ status of the stomatal complex and it has been concluded that ABA acts directly on guard cells in effecting stomatal closure^{8,10,11}. There is, however, little information about the distribution of ABA in specific cells of the epidermis or about the sensitivity of the stomatal complex to ABA. We present here evidence that ABA can accumulate in the stomatal complex of *Commelina communis* L., a species used extensively in studies of stomatal physiology¹². The leaves of this plant yield relatively uncontaminated epidermal strips¹³ and this property has made it possible to calculate the apparent sensitivity of the stomatal complex to ABA. The evidence is based on experiments involving the uptake, distribution and metabolism of exogenous $2\text{-}^{14}\text{C}$ -ABA.

Figure 1 shows that during 1 h when $2\text{-}^{14}\text{C}$ -ABA was supplied through the transpiration stream there was a substantial increase in the radioactivity present in the lower epidermis. Stomatal closure was complete 9 min after application of ABA. These data enable us to calculate an upper limit

times necessitating the use of extremely high specific activity labelled ABA are required. (2) The guard cells seem to be metabolically the most active cells in the epidermis¹⁵ and could be acting as metabolic sinks, although this does not necessarily deny a role for ABA in closure. ABA accumulation in guard cells may represent a form of compartmentalisation or sequestration away from active sites¹⁷. (3) There is evidence that ABA

affects solute distribution between epidermal cells and guard cells¹⁸, but the precise intracellular sites of ABA action remain to be elucidated.

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C. ITAI*

Department of Biology,
University of Stirling, UK

J. D. B. WEYERS
J. R. HILLMAN

Department of Botany,
University of Glasgow, UK

H. MEIDNER
C. WILLMER

Department of Biology,
University of Stirling, UK

Received 31 August; accepted 25 November 1977.

*Present address: Ben Gurion University of the Negev, Beer-Sheva, Israel.

1. Raschke, K. A. *Rev. Plant Physiol.* 26, 309–40 (1975).
2. Mizrahi, Y., Scherings, S. G., Malis Arad, S. & Richmond, A. E. *Physiol. Plogia* 31, 44–50 (1974).
3. Mittelheuser, C. J. & Van Steveninck, R. F. M. *Nature* 221, 281–282 (1969).
4. Tucker, D. J. & Mansfield, T. A. *Planta* 98, 157–163 (1971).
5. Wright, S. T. C. & Hiron, R. W. P. *Nature* 224, 719–720 (1969); Walton, D. C., Harrison, M. A. & Cote, P. *Planta* 131, 141–144 (1976).
6. Loveys, B. R. *Physiol. Plogia* 40, 6–10 (1977).
7. Hiron, R. W. P. & Wright, S. T. C. *J. exp. Bot.* 24, 769–781 (1973).
8. Horton, R. F. & Moran, L. Z. *Pflanzenphysiol.* 66, 193–196 (1972).
9. Mansfield, T. A. & Jones, R. J. *Planta* 101, 147–158 (1971).
10. Horton, R. F. *Can. J. Bot.* 49, 583–585 (1970).
11. Raschke, K. *Planta* 125, 243–259 (1975).
12. Willmer, C. M. & Mansfield, T. A. *New Phytol.* 68, 363–375 (1969).
13. Willmer, C. M., Pallas, J. E. & Black, C. C. *Plant Physiol.* 52, 448–452 (1973).
14. Kriedemann, P. E., Loveys, B. R., Fuller, G. L. & Leopold, A. C. *Plant Physiol.* 49, 842–847 (1972).
15. Meidner, H. & Willmer, C. *Curr. Adv. Plant Sci.* 17, 1–15 (1975).
16. McWha, J. A. & Hillman, J. R. *Stain Technol.* 48, 136–138 (1973).
17. Cummins, W. R. *Planta* 114, 159–167 (1973).
18. Itai, C. & Meidner, H. *Nature* 271, 653–654 (1978).

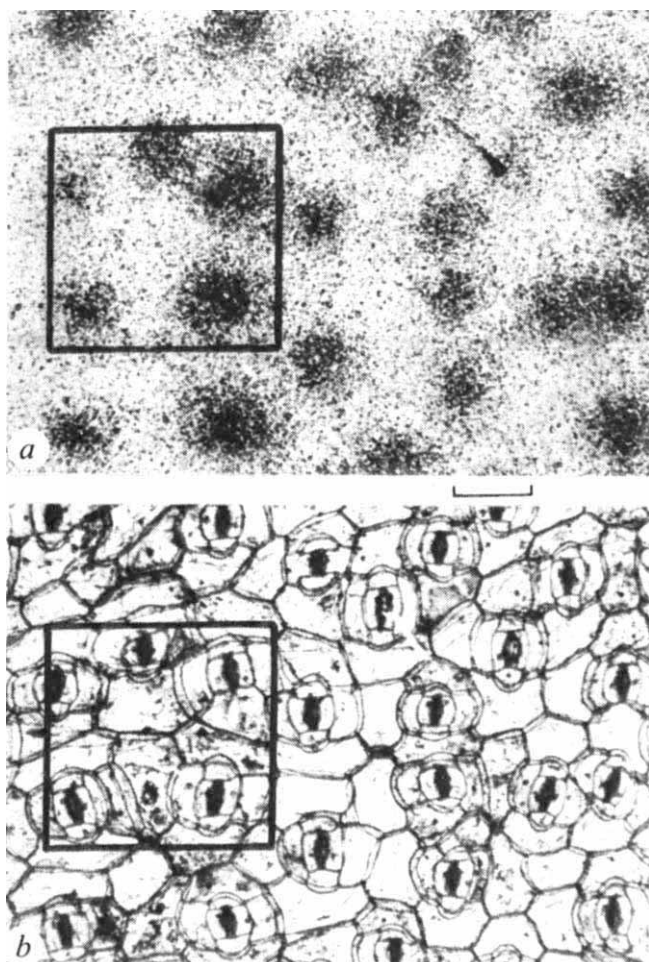


Fig. 2 Distribution of radioactivity in lower epidermis tissue of *C. communis* leaves after floating on $2\text{-}^{14}\text{C}$ -ABA solution for 1 h. *a*, Aggregation of silver grains in the photographic emulsion. *b*, Position of guard cells in the associated freeze-dried *Commelina* epidermis (neutral red stained). 50-mm^2 squares of abaxial epidermal tissue were taken from mature leaves of non-flowering shoots. These were floated for 1 h, cuticle uppermost, on 1 mM CaCl_2 solution containing $2\text{-}^{14}\text{C}$ -ABA ($4\text{ }\mu\text{Ci ml}^{-1}$, 11.1 Ci mol^{-1}) maintained at $27\text{ }^\circ\text{C}$ in a water bath. This solution was gently shaken. After incubation the tissue was rinsed five times for 20 s, with agitation, in 1 ml 1 mM CaCl_2 . Optional prestaining in an aqueous neutral red solution was carried out for 1 min before this. Open stomata were obtained as previously described and closed stomata by floating leaves on water in the dark. Thin-layer chromatography in four solvent systems (benzene:acetic acid, 70:30:1; ethyl acetate:toluene:acetic acid, 1:10:2; chloroform:methanol, 1:1; *n*-hexane:ethyl acetate, 1:1) was used to estimate radiochemical purity of methanol:ether extracts of epidermal tissue incubated in $2\text{-}^{14}\text{C}$ -ABA solution for 1 h. Efflux of radioactivity was estimated by floating such tissue, cuticle uppermost, on 1 ml of 1 mM CaCl_2 solution for 90 min and estimating the radioactivity present in the epidermis and efflux medium. The autoradiographic procedure was a modification of that used by Willmer *et al.*¹³. Epidermal tissue was applied, cuticle uppermost, to a subbed slide and immersed in liquid nitrogen before freeze-drying for approximately 12 h. The emulsion side of Kodak Pan-F film was applied directly to the tissue and held in place with another slide which was then taped to the first. The film was exposed for 3 weeks in a desiccator at $-15\text{ }^\circ\text{C}$ before development. Controls for pressure artefacts and chemography¹⁶ proved negative scale bar, $100\text{ }\mu\text{m}$.

Effect of abscisic acid on solute transport in epidermal tissue

An important aspect of the stomatal mechanism in green plants is the transport of solutes into and out of guard cells in response to stimuli such as illumination¹. The effect of abscisic acid (ABA) in causing stomata to close has been ascribed to ABA-promoted solute leakage from guard cells². The experiments reported here show that leakage alone is insufficient to explain stomatal closure caused by ABA. The involvement of live epidermal cells, acting as recipients of solutes from guard cells, is necessary, as Penny and Bowling have suggested, especially when considering intact leaves³.

To test the requirement for live epidermal cells in ABA-mediated stomatal closure, epidermal strips of *Commelina communis* without living epidermal cells were floated on radioactive ABA in solution. The stomata remained open, but autoradiographs of the strips clearly showed that ABA had accumulated in the stomatal complexes as in untreated strips⁴ (Fig. 1).

We used two approaches to examine the effects of ABA on solute distribution between epidermal and guard cells. Neutral red staining of epidermal strips showed that treatment with ABA resulted in a diminished accumulation of stain in guard cells, and increases in both the rate of accumulation and the final concentration in live epidermal cells. Staining showed that where stomata were adjacent to ruptured epidermal cells the guard cells remained open after application of ABA (Fig. 2). If the transport of neutral red can be taken as an indication of solute transport, one can conclude that in ABA-treated tissue epidermal cells function as active(?) sinks for solutes from guard cells.

For a direct assessment of solute concentrations in epidermal cells, epidermal strips were treated or not treated with ABA while turgor was kept at the optimum epidermal water deficit^{5,6} by floating the tissue on mannitol solution.

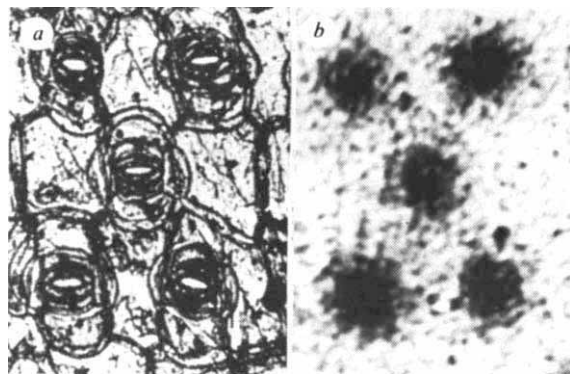
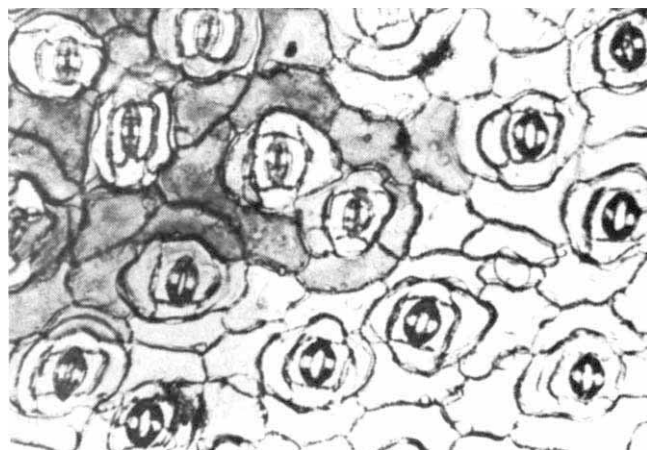


Fig. 1 *a*, Photomicrograph of an epidermal strip of *Commelina communis* with stomata open. The strip had been illuminated while floating on a buffer solution at pH 4.5 to kill the epidermal cells⁷. When stomata had remained open at a steady state aperture for 20 min the strip was transferred for 60 min to a solution of 1 μ Ci DL-*cis-trans*-2-¹⁴C-ABA (11.7 Ci mol⁻¹). Stomata remained open although ABA had accumulated in stomatal cells as in (*b*). *b*, Autoradiograph of the strips shown in (*a*). Accumulation of ABA in the stomatal cells did not cause stomatal closure.

Under the influence of ABA there was an increase in the concentration of solutes in the epidermal cells probably functioning as recipients of solutes from guard cells. This resulted in a striking difference in the incidence of plasmolysis. In tissue floating on 0.2 M mannitol without ABA almost all epidermal cells were plasmolysed and stomata opened to the maximum extent. In tissue treated with ABA very few epidermal cells were plasmolysed and stomata were closed. This confirmed the indication, gained from neutral red staining, that solutes from guard cells accumulated in adjacent epidermal cells, and of necessity raised their turgor pressure. It could be concluded, however, that the latter played only a secondary role in ABA-mediated stomatal closure because stomata adjacent to slightly plasmolysed epidermal cells nevertheless closed in the tissue treated with ABA.

Although these experiments have clarified part of the mechanism of ABA action on the stomatal complex as far

Fig. 2 Photomicrograph of a microscope field of view ($\times 250$) of an epidermal strip of *Commelina communis*. Stomata had been opened in illuminated leaves kept in CO₂-free air. ABA was supplied in the transpiration stream for 10 min. Epidermal strips were then taken and stained for 1 min by floating on neutral red (1:20,000), after which they were mounted in liquid paraffin. From the staining it was clear which epidermal cells had been ruptured and which had remained intact. Measurements were made of the effect of ABA on stomatal aperture, the rate of accumulation of dye in the different cells and the final intensity of stain in epidermal and guard cells. Rate of accumulation was measured by photographic recording on colour film. Intensity of stain was measured by transmittance at 600 nm in a Reichert Zetopan using the colour films obtained. Only those stomata which were adjacent to live (stained) epidermal cells had closed.



as solute distribution is concerned, the precise site of that action remains to be demonstrated. The effect could be restricted to the guard cells or may extend to other epidermal cells, especially the subsidiary cells whose exact role in *Commelina communis* is not fully known.

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C. ITAI*

HANS MEIDNER

Department of Biology,
University of Stirling, UK

Received 16 September; accepted 19 December 1977.

* Present address: Department of Biology, Ben Gurion, University of the Negev, Israel.

1. Zeiger, E., Moody, W., Hepler, P. & Varela, F. *Nature* **270**, 270–271 (1977).
2. Dittich, P. & Raschke, K. *Planta* **134**, 77–81 (1977).
3. Penny, M. G. & Bowling, D. J. F. *Planta* **119**, 17–25 (1974).
4. Itai, C., Weyers, J. D. B., Hillman, J. R., Meidner, H. & Willmer, C. *Nature* **271**, 652–653 (1978).
5. Glinka, Z. *Physiol. Pl.* **24**, 476–479 (1971).
6. Edwards, M., Meidner, H. & Sheriff, D. W. *J. exp. Bot.* **27**, 163–171 (1976).
7. Squire, G. R. & Mansfield, T. A. *New Phytol.* **71**, 1033–1043 (1972).

Characterisation of the surface coat of *Trypanosoma congolense*

THE protozoan parasites *Trypanosoma congolense*, *T. brucei* and *T. vivax* cause extensive disease problems to livestock in Africa¹. In many cases trypanosomiasis results from mixed infections due to all three species². Immunisation has not been successful because of the ability of the parasites to evade the host immune response by changing the antigenic structure of the surface coat³. Chemical and immunochemical characterisation of the surface coat of all three species should give insight into the mechanism of antigenic variation. Recent work on the surface coat of *T. brucei* demonstrates that antigenic variation is the result of a change in structure of a single, variant-specific, surface glycoprotein^{4,5}. No comparable information is available on the structure of the surface coat of *T. congolense* and *T. vivax*. We report here some properties of the surface coat of *T. congolense*.

An isolate⁶ of *T. congolense* (Transmara strain) was supplied by the Kenya Veterinary Laboratories. Clone X4 was derived from the Transmara strain by injecting a single trypanosome into lethally irradiated (900 R) mice. Trypanosomes of this clone were normally grown in irradiated mice and separated from infected blood⁷. Occasionally organisms were collected from normal rats after an additional 3 d passage with no change in surface antigen observable by analytical isoelectrofocusing (IEF), or immunofluorescence using a variant-specific antiserum.

Uncloned or cloned parasites were labelled by lactoperoxidase-catalysed ¹²⁵I iodination or by galactose oxidase oxidation followed by NaHB₄ (³H) reduction. Both enzymes are known to be unable to penetrate the lipid bilayer, and therefore the two methods are commonly used to detect exposed glycoproteins⁸. The adaptation of these labelling procedures to trypanosomes (see legends to Figs 1 and 2) allowed the analyses of surface coat presented here and provided markers for purification of surface material. The labelled coat was extracted by repeated freezing and thawing followed by centrifugation at 230,000g (60 min.). The presence of 1 mM *N*-tosyl-L-lysyl-chloromethane (TLCK) prevented proteolytic breakdown of the labelled material at least during separation into supernatant and pellet fractions at 4° C.

Between 60 and 80% of total radioactivity was present in the soluble supernatant fluid. Most of the remaining radioactivity was solubilised by treating the 230,000g pellet with nonionic detergent NP-40 (Shell) at 0.5% v/v for 30 min at

0 °C and centrifuging at 230,000g (60 min). Total labelled parasites and both aqueous- and detergent-solubilised supernatant fluids were analysed by 7.5–15% gradient polyacrylamide-sodium dodecyl sulphate gel electrophoresis (SDS-PAGE) in reducing and non-reducing conditions¹¹.

Gels were stained with Coomassie blue and destained before detection of radioactive components either by counting 2-mm gel slices or by autoradiography¹². Although a large number of proteins were stained by Coomassie blue throughout the gel length only one major peak containing both ¹²⁵I and ³H was consistently detected by SDS-PAGE of total parasites and soluble or insoluble extracts. The appearance of this peak was unchanged whether the preparations were derived from cloned or uncloned trypanosomes (Figs 1 and 2). The apparent molecular weight of the glycoprotein is 56,000. Gels run in non-reducing conditions gave similar results, and no dimers or oligomers were detectable. A few minor labelled peaks accounting for 1 to 5% of the total radioactivity were also detected by SDS-PAGE and are being analysed. The galactose oxidase catalysed labelling

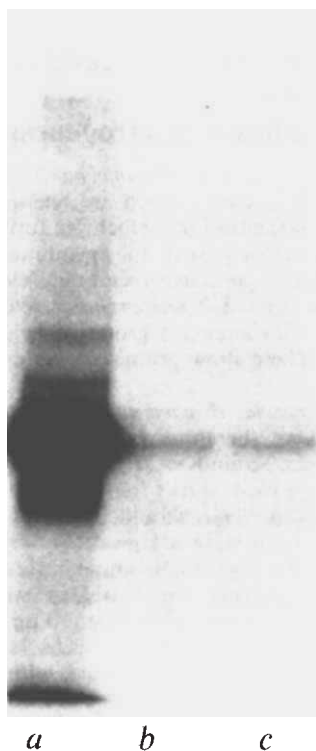


Fig. 1 Autoradiograph patterns of ¹²⁵I *T. congolense* clone X4 surface proteins. *a*, 10⁷ Parasites solubilised with 2% SDS; *b*, water soluble 230,000g supernatant fluid and *c*, NP-40 soluble 230,000g supernatant fluid from the same number of parasites. The autoradiography shows that the sum of radioactivity in *b* and *c*, does not equal *a*, probably because of loss of material which occurs in separating the major labelled glycoprotein into water soluble and detergent soluble supernatant fluids; in addition a small portion of the radioactivity remains with the detergent insoluble pellet (not shown). The SDS-PAGE slab is 7.5%–15% acrylamide, stabilised with 10% glycerol in the 15% solution. Apparent molecular weights were determined using calibration curves obtained from protein standards subjected to electrophoresis on the same slab gel (see legend to Fig. 2). The method of Marchalonis *et al.*⁸ for enzymatic radioiodination of leukocytes was modified as follows: 2 × 10⁸ washed trypanosomes were resuspended in 50 µl 0.01M phosphate saline buffer, 1% glucose, pH 7.4 (PGS pH 7.4) containing 20 µg of lactoperoxidase (Sigma) and 250 to 500 µCi of Na¹²⁵I (Amersham). The reaction was initiated by the addition of 20 µl of 0.03% H₂O₂ and terminated after 5 min by adding a 100 times excess of PGS pH 8.0 (ref. 7). Two subsequent washes were carried out with the same buffer containing 1% foetal calf serum. Parasite motility was unimpaired at the end of the procedure.

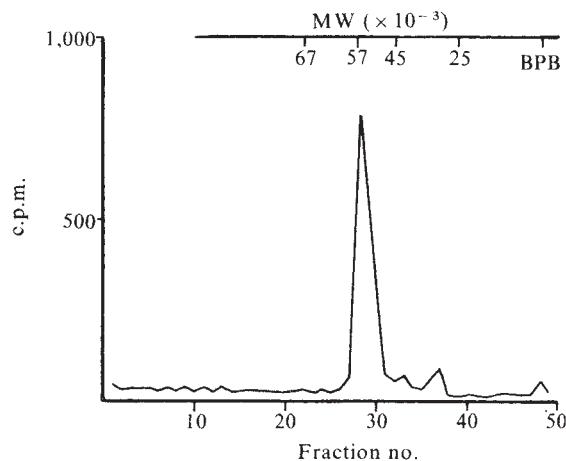


Fig. 2 SDS-PAGE in reducing conditions of 230,000g supernatant fluid from uncloned *T. congolense*. Organisms were labelled on galactose and galactosamine(s) by galactose oxidase oxidation and NaBH₄ (³H) reduction. Molecular weight markers were: albumin (67,000), human IgG heavy chains (57,000), ovalbumin (45,000), human IgG light chains (25,000), bromophenol blue (BPB). ³H labelling of carbohydrates was achieved by modifying the method of Gahmberg⁹. Trypanosomes (2 × 10⁸) in 1 ml PGS pH 7.4 (buffer reference in legend to Fig. 1) were incubated with 1 unit galactose oxidase (Worthington) and 500 units catalase (Worthington) for 30 min. The parasites were then washed with PGS pH 8, resuspended in 1 ml of PGS pH 7.4 and NaBH₄ (³H) 6 Ci mmol⁻¹ (Amersham) was added to give a final concentration of 1 mM and a specific activity of 5mCi per 2 × 10⁸ trypanosomes. The reduction was carried out at room temperature for 15 min with occasional shaking. Termination of the reaction and washes of parasites were as for the iodination method (legend to Fig. 1). Parasites retained normal motility.

technique was used mainly to show the presence of carbohydrate moieties on the surface coat of *T. congolense*. We now routinely use only surface iodination for tracing the parasite coat.

Parallel studies carried out with *T. brucei* (LUMP 227) confirmed previous work showing that the surface coat consists of one major glycoprotein¹. The apparent molecular weight of the *T. brucei* variant antigen is 62,000. A sample of purified variant antigen from *T. brucei* clone 052 (ref. 4) donated by G. A. M. Cross showed the same molecular weight when measured by the gradient SDS-PAGE technique. In *T. brucei* we also observed the same amount of insoluble glycoprotein as in *T. congolense*. The small difference in apparent molecular weight between the major surface glycoprotein of *T. congolense* and *T. brucei* could have one of several explanations, for example, a peptide bond of the *T. congolense* antigen might be subject to specific proteolytic degradation, or the extent of glycosylation might be very different in the two species.

Supernatant fractions of iodinated cloned and uncloned *T. congolense* were analysed by IEF in polyacrylamide gels. Uncloned material showed many peaks ranging in *pI* from 5 to 8. It is known that the surface coat glycoprotein of *T. brucei* is variant specific and in fact iodinated supernatant fluids from various *T. brucei* clones always showed one homogeneous peak with a specific isoelectric point (for example Fig. 3b). One might expect similar results for *T. congolense*; however, three peaks were resolved by IEF of a labelled supernatant fraction from clone X4 of *T. congolense* (Fig. 3a). Preparative purification of the surface glycoprotein from 2 × 10¹⁰ cloned parasites⁴ yielded about 1 mg of each of the three components (*pI* 6.2, 6.4 and 6.6).

An antiserum prepared in rabbit against the IEF peak of *pI* 6.6 stained > 99% of the parasites using immunofluorescence and showed a single precipitin line in immunodiffusion. Both aqueous- and detergent-soluble supernatant fluids were tested in immunoprecipitation as follows: supernatant fluids derived from 10⁸ parasites were incubated with

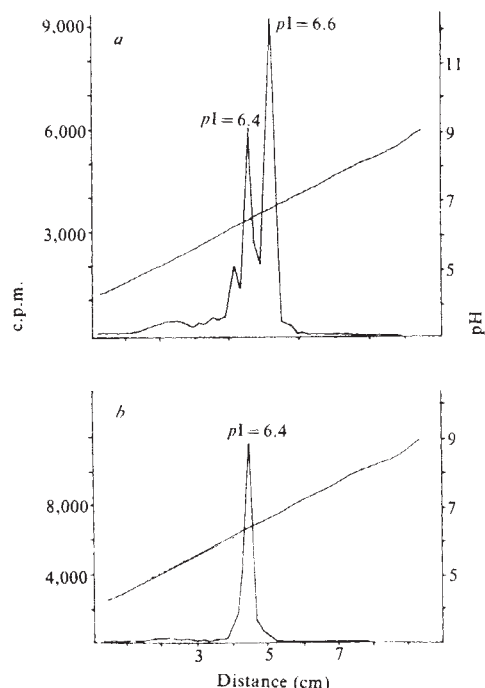


Fig. 3 *a*, A radioiodinated clone of *T. congolense* (X4) was freeze-thawed three times in water and separated into 230,000g (60 min) supernatant and pellet fractions. The supernatant fluid was dialysed against water at 4 °C then run across the width of a standard polyacrylamide gel isoelectrofocusing plate (pH 3.5–9.5) supplied by LKB. The gel was stained for protein with Coomassie blue and destained in ethanol–water–acetic acid (6:13:1). Radioactivity measurements were made on 2 mm fractions. (*b*), As (*a*) except that the radioiodinated trypanosomes were a clone of *T. brucei*.

rabbit anti-variant specific antiserum or normal rabbit serum as control for 30 min at room temperature. An optimal amount of sheep anti-rabbit immunoglobulin serum was added and the mixtures left at 4 °C overnight. Precipitates were washed three times with buffered saline, dissolved in 0.08 M Tris pH 6.8, 0.1 M dithiothreitol, 2% SDS and boiled for 5 min before electrophoresis. SDS-PAGE analysis of the specific precipitates showed the same 56,000 radioactive peak seen in Figs 1 and 2, as well as the protein with pI 6.4 which has a lower molecular weight (data not shown). No radioactive material was precipitated by normal rabbit serum.

These data suggest that an antigenically homogeneous clone of *T. congolense* yields a single specific glycoprotein as surface antigen and that the portion which is detergent-solubilised is immunologically indistinguishable from the soluble glycoprotein, although it possibly has a different attachment to the trypanosome plasma membrane. This might reflect a precursor–product relationship.

The heterogeneity of surface-labelled glycoprotein from cloned *T. congolense* observed from IEF is likely to be due to proteolytic degradation. Breakdown of the 56,000 molecular weight protein was observed even during separation of broken cells into supernatant and pellet fractions at 4 °C in the absence of TLCK. The extent of degradation was enhanced by any further purification procedures, and the final yield of purified variant antigen is far from satisfactory. Improvements of the solubilisation and purification procedures are in progress and will be published elsewhere.

There are several overall similarities between the surface coats of *T. congolense* and *T. brucei*. In both cases one glycoprotein is predominantly accessible to surface labelling; the distribution of surface glycoprotein between water soluble and insoluble fractions of broken cells is similar, as are the molecular weights. These results suggest the need

for comparable studies on *T. vivax* to provide a more unified understanding of basic surface coat structure in African trypanosomes.

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L. ROVIS
A. F. BARBET
R. O. WILLIAMS

International Laboratory for Research on Animal Diseases,
PO Box 30709,
Nairobi, Kenya

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1. Fiennes, R.N.T.W. in *The African Trypanosomiasis* (ed. Mulligan, H. W.) 729–750 (Allen & Unwin, 1970).
2. Wilson, A. J., Paris, J. & Dar, F. K. *Trop. Anim. Health Prod.* 7, 63–71 (1975).
3. Vickerman, K. *Ciba Fdn Symp.* 25, new ser. 53–70 (1974).
4. Cross, G. A. M. *Parasitology* 71, 393–417 (1975).
5. Bridges, P. J., Cross, G. A. M. & Bridges, J. *Nature* 263, 613–614 (1976).
6. Welde, B. T., Lozsch, R., Deindl, G., Sadun, E. H., Williams, J. S. & Warui, G. *Exptl Parasit.* 36, 6–19 (1973).
7. Lanham, S. M. & Godfrey, D. G. *Exptl Parasit.* 28, 521–524 (1970).
8. Marchalonis, J. J., Cone, R. E. & Santer, V. *Biochem. J.* 124, 921–927 (1971).
9. Gahmberg, C. G. & Hakomori, S. *J. biol. Chem.* 248, 4311–4317 (1971).
10. Hunt, R. C. & Brown, J. C. *Biochemistry* 13, 22–28 (1974).
11. Laemmli, U. K. *Nature* 227, 680–685 (1970).
12. Fairbanks, G., Levinthal, C. & Reeder, R. H. *Biochem. biophys. Res. Commun.* 20, 393–399 (1965).

Behavioural mutants of *Paramecium caudatum* with defective membrane electrogenesis

THE swimming behaviour of *Paramecium* is mostly dependent on the activity of its cilia¹, which are regulated by the intracellular cation concentration, which in turn is modified by Ca-dependent electrogenesis in the membrane^{2,3}. Behavioural abnormalities due to defective membrane electrogenesis have been found in mutants of *P. tetraurelia*^{4–7}. We have now found similar abnormalities among a group of behavioural mutants of *P. caudatum*. These show promise of an excellent source of genetic markers.

Unlike *P. tetraurelia*, *P. caudatum* does not exhibit natural autogamy⁸, and is therefore a less convenient source of homozygous clones. Strain Kyk201 (mating type VI of syngen 3), however, which we used, shows frequent selfing conjugation so that recessive mutant genes should be expressed. The progeny of selfing conjugations show a high rate of survival.

P. caudatum in the logarithmic phase in fresh lettuce medium with *Klebsiella aerogenes*⁹ were treated with the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (10 µg ml⁻¹) for 40 min and then cloned. After the isolated cells had divided 12–15 times, clones were starved to induce selfing conjugation¹⁰. Those containing many conjugating pairs were fed again with bacterised medium to induce five to eight more cell divisions. Then the progeny were subjected to galvanotactic and geotactic¹¹ screening. Every cell which showed abnormal swimming behaviour in an electric field was pipetted into a separate dish to make an independent clone. The subsequent progeny were compared with the wild type with respect to swimming behaviour in response to various stimuli and with respect to electrophysiological characteristics.

The wild type makes an avoiding response due to a transient ciliary reversal when it encounters an obstacle. It may also do this spontaneously. It makes repetitive avoiding responses at fairly regular intervals in a mixture of Ba²⁺ and Ca²⁺ (ref. 12) ('barium dance', Fig. 1*a*), and undergoes longer term ciliary reversal (about 1 min) when K⁺ is above a certain concentration in the external medium¹³. Depolarisation of the membrane by an outward electric current causes ciliary reversal in the wild type¹⁴. This reversal is always associated with an inflow of Ca²⁺ through the membrane due to an increase in Ca permeability^{2,3}. Ca is a mediator for the coupling of membrane permeability with ciliary reversal¹⁵.

As summarised in Table I, none of these behavioural responses due to ciliary reversal was seen in mutant 13B

Table 1 Swimming behaviour and ciliary responses to various stimuli in mutants of *P. caudatum*

Strain	Swimming velocity (μms^{-1})	Avoiding response	Escape response	Ciliary reversal by outward current	Ciliary augmentation by inward current	Ciliary reversal by K^+ (s)	Barium* dance
Wild type							
Kyk201	1384 $\pm$ 27	+	+	+	+	43.6 $\pm$ 4.8	+
Mutants							
13B (CNR)	1548 $\pm$ 27	—	+	—	+	0	—
13D (K^+ sensitive)	1108 $\pm$ 32	+	+	+	+	255.6 $\pm$ 31.0	+
11C (slow swimmer)	864 $\pm$ 16	+	+	+	+	26.3 $\pm$ 6.1	+

+ and —, Presence and absence of the response respectively. All measurements were made at room temperature (20–25 °C). Swimming velocity was measured by Dryl's method¹² in a mixture of 4 mM K^+ , 1 mM Ca^{2+} and 1 mM Tris-HCl (pH 7.2). The duration of ciliary reversal was measured when the specimen was transferred from a mixture of 1 mM K^+ , 1 mM Ca^{2+} and 1 mM Tris-HCl (pH 7.2) to a mixture of 20 mM K^+ , 1 mM Ca^{2+} and 1 mM Tris-HCl (pH 7.2). The figures represent the average and standard error of 10–20 measurements in different specimens.

* Repetitive avoiding response in association with all-or-none type action potentials shown in a mixture of Ba^{2+} and Ca^{2+} .

(Fig. 1b). It showed no ciliary reversal in response to various stimuli. Electrophysiological examination revealed that, in contrast to the wild type^{16,17}, it produced no regenerative Ca action potential in response to a depolarising current (Fig. 2a and c). Only a residual active Ca^{2+} response was detectable when the current was very strong and the potential response was displayed in a form of phase plane trajectory¹⁸ (Fig. 3a, b, d and e). However, ATP-reactivated cilia of Triton-extracted specimens of 13B (which have a disrupted cell membrane) were reversed in their beating orientation when the Ca^{2+} concentration in the reactivation medium was raised above $5 \times 10^{-6}\text{M}$, as happens with similarly treated wild type specimens^{15,19}. Externally applied Ca has direct access into such cells and can influence the Ca^{2+} -sensitive reversal mechanism of cilia without restriction by the membrane. Thus, the reversal mechanism of the cilia is functional in mutant 13B, and the behavioural deficiency is probably due to a defect in the depolarisation-sensitive Ca gating system in the membrane. We shall now refer to mutant 13B as CNR (caudatum non-reversal).

A transient increase in forward swimming speed due to increased beat frequency of the cilia in the normal direction (ciliary augmentation) was observed in CNR, as in the wild type, in association with a hyperpolarising mechanoreceptor potential after mechanical stimulation of the posterior membrane²⁰ (Table 1, Fig. 2f and h). An injection of inward current into a CNR cell always caused ciliary augmentation in

association with an increase in K^+ permeability of the membrane, as in the wild type²¹ (Table 1, Fig. 2b and d, Fig. 2c and f). Thus both the mechanosensitive K gating system in the posterior membrane and the hyperpolarisation-sensitive K gating system in the general membrane remain intact in the CNR specimen.

Mechanical stimulation of the anterior membrane of a CNR cell induced a transient depolarising mechanoreceptor

Fig. 2 Responses of the membrane potential to constant current stimulation (a–d) and mechanical stimulation (e–h) in *P. caudatum*. a, b, e and f, Wild type specimen; c, d, g and h, CNR mutant. E_m , membrane potential: dashed lines show the resting level, and upward and downward deflections correspond to depolarisation and hyperpolarisation of the membrane, respectively. S_i , transmembrane current: dashed lines show zero current level, and upward deflection means outward current while downward deflection means inward current. S_m , deflection in the trace shows the duration and relative intensity of the electric pulse activating a piezoelectric crystal to drive a glass rod against the surface of the cell. The horizontal line in the centre of the figure corresponds to 20 ms in a, b, c and d, and to 40 ms in e, f, g and h. The vertical line corresponds to 20 mV for E_m , 4 nA for S_i and to 2 V s^{-1} for E_m .

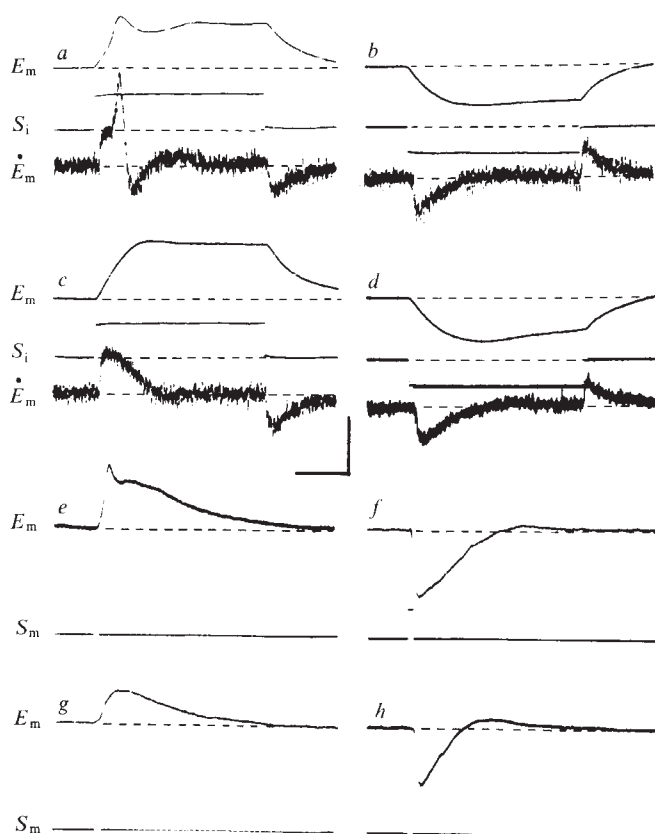


Fig. 1 Swimming behaviour of *P. caudatum* in a mixture of 1 mM BaCl_2 and 1 mM CaCl_2 (buffered to pH 7.2 by 1 mM Tris-HCl). The photographs were taken under dark-field illumination with an exposure time of 4 s (room temperature 25 °C). a, Wild type specimens (strain Kyk201) show repetitive avoiding response, which makes the specimen follow a zig-zag or star-shaped swimming path (barium dance). b, CNR mutants (strain 13B) do not dance, but continue to swim forward following a long-pitched, spiral path similar to that followed by the unstimulated wild type. The bar corresponds to 1 mm.

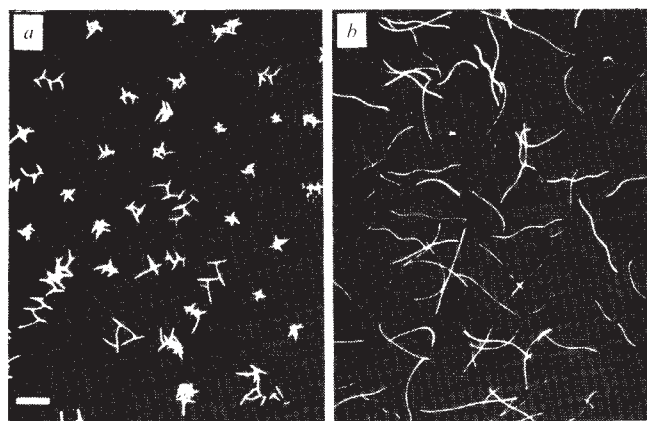


Table 2 Cross breeding analysis between wild type and behavioural mutants in *P. caudatum*

Cross	Generation	Phenotype mutant	wild type	χ^2 and P	Survival (%)
13B $\times$ Kyky 1	F ₁	0	85	$\chi^2 = 0.01$ $P = 0.9-0.95$	60.7
	F ₂ Observed	4	15		22.6
	F ₂ Expected	4.8	14.2		
11C $\times$ Kyky 1	F ₁	0	28	$\chi^2 = 0.10$ $P = 0.7-0.8$	20.0
	F ₂ Observed	10	25		27.6
	F ₂ Expected	8.8	26.2		
13D $\times$ Kyk201	F ₁ Observed	11	17	$\chi^2 = 0.64$ $P = 0.3-0.5$	66.7
	F ₂ Observed	14	14		
	F ₂ Expected	14	14		

potential, which, as expected, was not followed by a regenerative Ca action potential of the general membrane (Fig. 2g). Weak reversal was observable only in the cilia on the mechanically stimulated anterior membrane. In wild type cells a regenerative Ca action potential was always accompanied by an anterior mechanoreceptor potential (Fig. 2e), caused by an increase in Ca^{2+} permeability on mechanical stimulation²². Thus the mechanosensitive Ca gating system in the anterior membrane is functional in CNR cells. Electro-genetic malfunction in the membrane of CNR is restricted to its voltage sensitive Ca gating system.

Mutant 13D showed remarkably long-lasting backward swimming, due to ciliary reversal, when transferred to K^+ -rich medium (about five times as long as for the wild type, Table 1). The resting membrane potential of 13D cells was much more depolarised than that of the wild type (about twice as much)

by an increase in the external K^+ concentration, especially in the lower concentration range (0.5–4.0 mM). The resting membrane of 13D therefore has a higher K^+ permeability than the wild type²³. We shall call 13D specimens ' K^+ -sensitive'. K^+ -sensitive showed a more marked increase in K^+ permeability than wild type in response to an inward current. This was demonstrated in a phase plane trajectory as a shift in the membrane e.m.f. toward the K^+ equilibrium potential (hyperpolarising direction) when the inward current stopped (compare Fig. 3h and c). Electrical responses of K^+ -sensitive to an outward current were essentially similar to those of the wild type (Fig. 3a and g).

Mutant 11C was distinctive for its slow swimming speed, being about half as rapid as the wild type (Table 1). Forward swimming of ATP-Mg²⁺-reactivated Triton-extracted cells of 11C was significantly slower than that of the wild type ($171.2 \pm 10.6 \mu\text{m s}^{-1}$ in 11C, $234.5 \pm 17.2 \mu\text{m s}^{-1}$ in the wild type at 25 °C)^{21,25}. This, together with the fact that the electrophysiological characteristics of the specimen were essentially similar to those of the wild type, indicates that the slower swimming of 11C is due primarily to some malfunction in its ciliary motile system. We shall call 11C 'slow swimmer'.

We investigated gene differences between the mutants and the wild type by cross breeding. As Table 2 shows, when CNR or slow swimmer was crossed to a wild type (Kyky 1), all F₁ progeny had wild type behavioural characteristics. Phenotypic segregation occurred in F₂ clones with an expected ratio of 1:3. Therefore the abnormal behaviour of CNR and slow swimmer is under the control of a single recessive gene²⁶, designated *cnrA* and *sl* respectively. In the case of K^+ -sensitive, the F₁ segregated phenotypically into those with K^+ -sensitive characteristics and those with wild type characteristics in a ratio of 1:1. Therefore we consider that the high K^+ sensitivity of K^+ -sensitive specimens is controlled by a dominant gene, K^+S .

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MIHOKO TAKAHASHI

Biological Institute,
Tohoku University,
Sendai, 980 Japan

YUTAKA NAITOH

Institute of Biological Sciences,
University of Tsukuba,
Ibaraki, 300-31 Japan

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- Jennings, H. S. *Behavior of the Lower Organism* (Columbia University Press, New York, 1906).
- Naitoh, Y. & Eckert, R. in *Cilia and Flagella* (ed. Sleight, M. A.) (Academic, London, New York, 1974).
- Eckert, R. *Science* **176**, 473–481 (1972).
- Kung, C. *Genetics* **79**, 423–431 (1975).
- Kung, C., Chang, S., Satow, Y., Van Houten, J. & Hansma, H. *Science* **188**, 898–904 (1975).
- Satow, Y. & Kung, C. *Nature* **247**, 69–71 (1974).

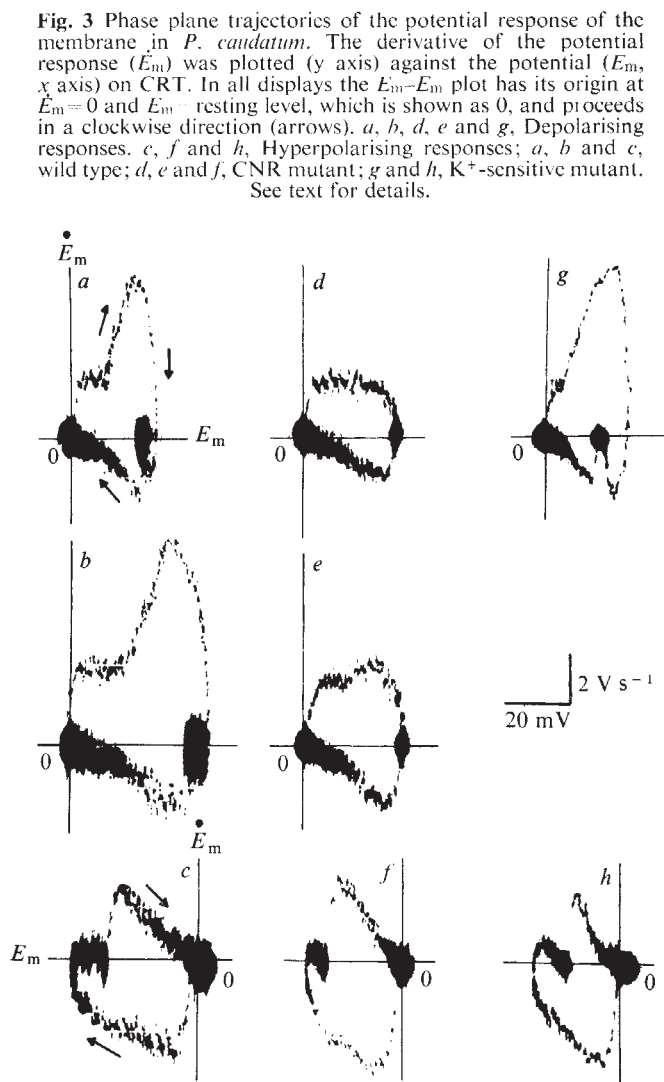


Fig. 3 Phase plane trajectories of the potential response of the membrane in *P. caudatum*. The derivative of the potential response ($\dot{E}_m$) was plotted (y axis) against the potential (E_m , x axis) on CRT. In all displays the $\dot{E}_m$ - E_m plot has its origin at $E_m=0$ and $\dot{E}_m$ resting level, which is shown as 0, and proceeds in a clockwise direction (arrows). a, b, d, e and g, Depolarising responses. c, f and h, Hyperpolarising responses; a, b and c, wild type; d, e and f, CNR mutant; g and h, K^+ -sensitive mutant. See text for details.

7. Kung, C. & Eckert, R. *Proc. natn. Acad. Sci. U.S.A.* **69**, 93–97 (1972).
8. Gilman, L. C. *Biol. Bull.* **80**, 384–402 (1941).
9. Hiwatashi, K. *Genetics* **58**, 373–386 (1968).
10. Hiwatashi, K. *Jap. J. Genet.* **35**, 213–221 (1960).
11. Kung, C. *Genetics* **69**, 29–45 (1971).
12. Dryl, S. J. *Protozool. Suppl.* **8**, 16 (1961).
13. Naitoh, Y. *J. gen. Physiol.* **51**, 85–103 (1968).
14. Eckert, R. & Naitoh, Y. *J. gen. Physiol.* **55**, 467–483 (1970).
15. Naitoh, Y. & Kaneko, H. *Science* **176**, 523–524 (1972); *J. exp. Biol.* **58**, 657–676 (1973).
16. Naitoh, Y. & Eckert, R. in *Experiments in Physiology and Biochemistry 5* (ed. Kerkut, G. A.) (Academic, London, New York, 1972).
17. Naitoh, Y., Eckert, R. & Friedman, K. J. *J. exp. Biol.* **56**, 667–681 (1972).
18. Jenerick, H. *Biophys. J.* **3**, 363–377 (1963).
19. Kung, C. & Naitoh, Y. *Science* **179**, 195–196 (1973).
20. Naitoh, Y. & Eckert, R. *Science* **164**, 963–965 (1969).
21. Naitoh, Y. & Eckert, R. *Z. vergl. Physiol.* **61**, 427–452 (1968).
22. Eckert, R., Naitoh, Y. & Friedman, K. J. *J. exp. Biol.* **56**, 683–694 (1972).
23. Satow, Y. & Kung, C. *J. exp. Biol.* **65**, 51–63 (1976).
24. Takahashi, M. *Zool. Mag., Tokyo* **85**, 366 (1976).
25. Naitoh, Y. *Zool. Mag., Tokyo* **85**, 367 (1976).
26. Takahashi, M., Naitoh, Y. & Eckert, R. *Jap. J. Genet.* **50**, 496 (1975).

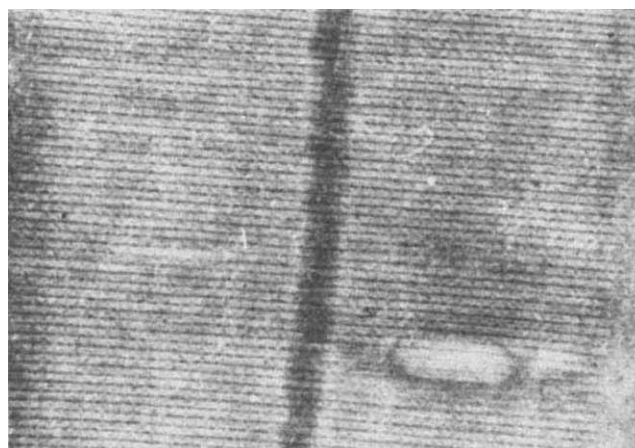


Fig. 1 STEM micrograph of unstained insect flight muscle section, original magnification $50,000\times$. This is a mixed-signal bright-field image, so that electron dense material appears dark. The central vertical dark region is the *M* band, the less dense vertical region to the right, the *Z* band. Scale bar, $0.2\ \mu\text{m}$.

Scanning transmission electron microscopy of unstained biological sections

THE scanning transmission electron microscope (STEM) has made possible the direct visualisation of single heavy atoms¹ and, by exploitation of the high dark-field detection efficiency, has been used successfully for the imaging of stained and unstained biological specimens². We report here some preliminary results which indicate that STEM can also be utilised for the imaging of unstained biological sections.

The use of STEM for biological electron microscopy has several advantages over conventional transmission electron microscopy. The irradiation dose is easily controlled by shifting the scanning raster to an adjacent area for focusing and stigmation and by the use of image storage devices (scan converters) and beam blanking. Simultaneous use of several imaging modes (for example, bright- and dark-field) combined with analogue or digital image-processing techniques can be used to enhance 'on-line' the contrast of difficult biological specimens as described in this paper. Additionally, microanalysis of easily identified specimen areas can be carried out *in situ* by energy-dispersive X-ray spectroscopy or electron energy-loss spectroscopy.

The material we have used for this work has been sectioned rigor flight muscle from *Lethocerus cordofanus*. This material was chosen because it has well defined structure, recognisable at both low magnification (the sarcomere repeat) and at higher magnification (the actin and myosin filaments and cross-bridges), and because an objective assessment of the image resolution can be made by the use of optical diffraction. The muscle was fixed with glutaraldehyde and embedded in araldite as described previously³, except that the osmium fixation and staining were omitted.

The microscopy was carried out on a V.G. Microscopes HB5 STEM operating at 100 kV with a cold field-emission source, an annular dark-field detector and a magnetic sector spectrometer as bright-field detector. Images were recorded directly from the photographic display screen on to 35 mm film which could be used without further processing for optical diffraction measurements. In order to minimise specimen dosage at high magnification, the microscope was used in a 'single shot' manner, that is, the image from a single scan at lower magnification was stored on a scan converter and redisplayed, with the electron beam blanked, in order to select a suitable area of specimen. The magnification was then increased and an area immediately adjacent to the selected region used for focusing. Finally, the scan raster position was shifted over to the appropriate position and the image recorded 'blindly' on film and simultaneously on the scan converter. The scan converter image was then redisplayed to assess various parameters, including the quality and correctness of focus of the recorded image. The total electron dose at the specimen was measured with a Faraday cage fitted directly above the specimen position.

To obtain sufficient contrast from a specimen where the contrast producing mechanism is the small difference in electron scattering between protein and the surrounding matrix of

embedding material, and to obtain an acceptable signal-to-noise ratio, it was necessary to use a mixed-signal mode of imaging. This had earlier been proposed for heavy atom imaging¹ and has been used for visualisation of unstained protein in ferritin particles⁴. The microscope was adjusted to give equal simultaneous signals in the dark-field and incoherent bright-field modes of operation. The two signals were then mixed in the algebraic mode $(\text{BF} - \text{DF})/(\text{BF} + \text{DF})$, and the mixed signal used to produce the final image. The resulting mixed signal has higher contrast for a given signal-to-noise ratio than either signal taken alone, and has the advantage that noise introduced by fluctuations in the electron beam is cancelled out by the division.

A typical image of unstained insect flight muscle is shown in Fig. 1. This was recorded with a 'single shot' after focusing on an adjacent area as described above, with a total electron dose, at an instrument magnification of $50,000\times$, of less than $25\ \text{electrons}\ \text{\AA}^{-2}$. The appearance of the thick and thin filaments is similar to that seen in stained sections and the cross-bridge structure can be seen weakly. The appearance of the *M* and *Z* bands is, however, different. The *M* band appears much darker than in stained sections, whereas the *Z* band, which stains very heavily, here appears to have a similar average density to that of the rest of the sarcomere. The appearance of the *Z* band was, in fact, somewhat variable, often being a little denser than the surrounding sarcomeres, but never appearing as dense as when seen in stained sections. It is not possible to say what these

Fig. 2 STEM micrograph, taken at magnification $100,000\times$. The dark region to the right is the *M* band. At this magnification, thick and thin filaments, and cross-bridges are visible. The narrow dark line to the left is the boundary of the previous scan. Loss of material has taken place to the left of this line, causing a distortion of the filament pattern.



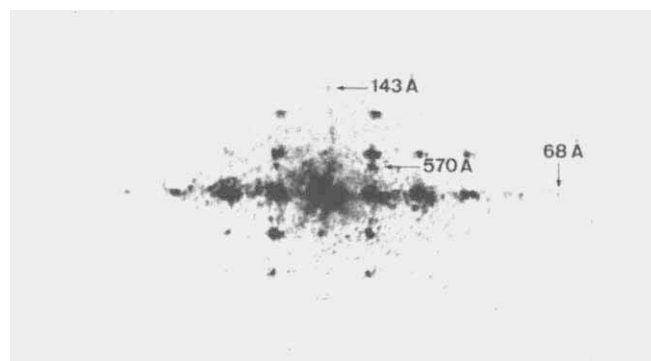


Fig. 3 Optical diffraction pattern taken from the image of Fig. 2. (The pattern has been rotated through 90° relative to the image.) There are strong reflections along the 380 Å and 190 Å layer lines, the 540 Å layer line is also clearly visible, and the 140 Å meridional reflections are weakly present.

density differences mean in terms of material present, but an attempt is being made to quantify the image density-mass thickness relationship.

Figure 2 shows an image taken at $100,000\times$ magnification, again by focusing and adjusting instrumental astigmatism off the area to be imaged. One of the main difficulties in working with unstained material is the very rapid loss of material (etching) which takes place during each scan. At the boundary between the two scans, there is a marked distortion of the filament pattern and a lower density in the region to the left that has been exposed to two consecutive scans. Some variation in density can be seen along the thin filaments, and cross-bridges can be distinguished quite clearly. An optical diffraction pattern of this image is shown in Fig. 3. The resolution in the equatorial direction extends to about 65 Å and in the meridional direction to about 148 Å. The overall resolution is close to that obtained for stained sections, and weaker intermediate layer lines⁵ were visible. The images shown in Figs 1 and 2 were of sections in the thickness range 600–800 Å. Work in progress on other materials indicates that good contrast can also be obtained for thinner sections.

These results indicate that for unstained biological material, conventionally fixed and embedded, resolutions approaching those obtained for stained sections (that is, the limit imposed by the preparative technique itself) can be obtained by STEM mixed-signal imaging. It is hoped that resolutions may be extended by improved specimen preparation methods, including cryo-techniques⁶, or in the case of periodic structures, by using low-dose imaging⁷. A further important advantage of the ability to image unstained material is the combination of this form of imaging with the use of STEM for X-ray microanalysis. Preliminary results indicate that secondary X-ray detection with a sensitivity at least a factor of 10 higher than that obtainable by conventional X-ray dispersive systems, and probe sizes down to a few angstrom units in diameter, is possible. The two techniques used together will provide a powerful tool for the characterisation of elemental distribution in biological materials.

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A. V. JONES
K. R. LEONARD

European Molecular Biology Laboratory,
Postfach 10.2209,
6900 Heidelberg,
FRG

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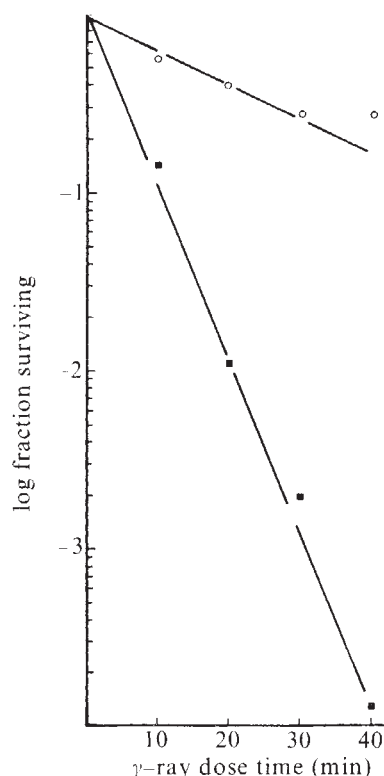
1. Crew, A. V. *Rev. Biophys.* **3**, 137–175 (1970).
2. Engel, A., Dubochet, J. & Kellenberger, E. *J. Ultrastruct. Res.* **57**, 322–330 (1976).
3. Bullard, B., Hammond, K. & Luke, B. M. *J. molec. Biol.* **115**, 417–440 (1977).
4. Langmore, J. *Proc. 6th Europ. Congr. on Electron Microscopy*, Vol. 1, 30–34 (1976).
5. Reedy, M. K. *J. molec. Biol.* **31**, 155, 176 (1968).
6. Taylor, K. A. & Glaeser, R. M. *J. Ultrastruct. Res.* **55**, 448–456 (1976).
7. Unwin, P. N. T. & Henderson, R. *J. molec. Biol.* **94**, 425–440 (1975).

Cellular glutathione is a key to the oxygen effect in radiation damage

OXYGEN is known to enhance biological changes induced by ionising radiation^{1,2}. The changes include chromosome breaks in *Tradescantia*³, mutation production in *Drosophila*, maize and bacteria^{3–6}, and rate of mitosis in grasshopper neuroblasts⁷. The biological and physical bases for the various enhancements remain obscure, and it has not yet been established that they are the result of a common mechanism. The quantitative aspects of the relationship between oxygen concentration and radiation response have been measured: for example, killing of *Escherichia coli* by X-ray radiation at a constant radiation dose⁸ and various interpretations of the quantitative aspects of the response base have been made^{9–12}. Ionising radiation has been proposed to interact with water to produce several products and these products interact with cellular material to produce the biological effects observed. In the reactions proposed, cellular sulphhydryl groups are supposed to be a primary cellular constituent which is reactive with the radiation products from water. We report here that one major cellular sulphhydryl constituent, glutathione (GSH), is apparently the major component in the interaction between radiation products and the cell, for cells unable to synthesise glutathione cannot be protected against killing by ionising radiation by reduction of the external oxygen concentration.

The response of GSH⁺ *E. coli* strain to irradiation in cellular suspension bubbled with air and argon (99.999% pure) is shown in Fig. 1. The isoefficiency dose ratio, that is, that dose ratio that gives equivalent survival in air and in the absence of oxygen⁹—now probably better known as oxygen enhancement ratio (OER), is about a factor of three. That is, in the presence of oxygen, ionising radiation is

Fig. 1 The oxygen effect with a GSH⁺ *E. coli* strain. ■, Survival in cell suspension bubbled in air; ○, survival of suspension bubbled for 10 min with argon. Cells grown in Penassay broth, washed, and irradiated in phosphate buffer, pH 6.9. Dose rate 2,230 rad min⁻¹ from a ⁶⁰Co source. Post-irradiation plating incubated at 30 °C.



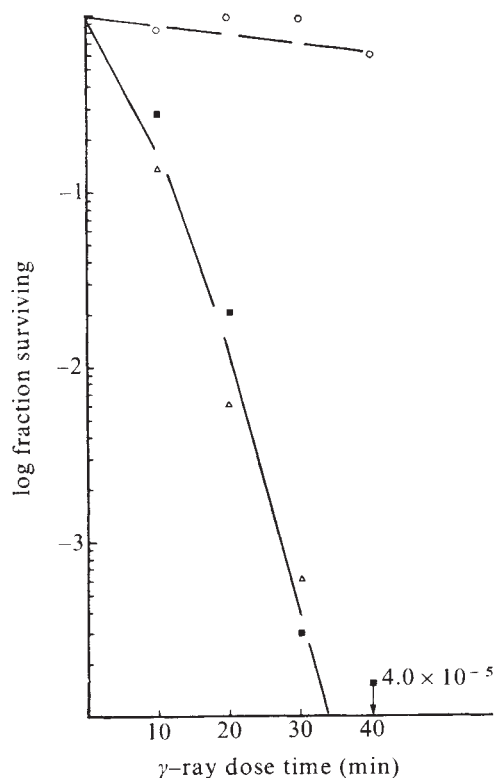


Fig. 2 The lack of a protective effect of argon treatment (oxygen removal) on a GSH⁻ *E. coli* mutant¹³. ○, GSH⁺ strain bubbled with argon; ■, GSH⁻ strain bubbled with argon; △, GSH⁻ strain bubbled with air. In this experiment, a spectinomycin-resistant derivative (20 µg ml⁻¹ spectinomycin) was selected in the GSH⁻ strain, and cells from a culture of it mixed with its spectinomycin-sensitive parent in buffer and irradiated. Plating on media with and without spectinomycin permits discrimination. Incubation at 30 °C. Dose rate, 2,230 rad min⁻¹.

about three times more effective than in its absence, or near absence.

A glutathione deficient mutant (GSH⁻)¹³ does not show the increased survival of its parent in the absence of oxygen (Fig. 2) and it is presumed that the response is related to its inability to synthesise glutathione. It should be noted that in the experiment of Fig. 2, a spectinomycin-resistant derivative mutant of the GSH⁻ strain was used in mixed suspension with its wild-type GSH⁺ parent: the mixed suspension irradiated and plated on media (±spectinomycin) permits counting of both GSH⁺ and GSH⁻ strains irradiated in the same suspension and in identical irradiation conditions.

The spectinomycin resistance of the GSH⁻ strain is not responsible for the radiation response, for its spectinomycin sensitive parent is not made more resistant to radiation by reduction of external cellular oxygen (data not shown).

Five experiments on the GSH⁻ mutant all gave the same result and clearly show that the GSH⁻ *E. coli* cells cannot be protected against radiation by oxygen removal.

A reversion of the GSH⁻ strain was selected on the basis of faster growth rate in broth than its parent, and the glutathione content of it, the parent, and the wild type measured by a modification of the procedure of Fuchs and Warner^{13,15}. The glutathione contents were (nmol per mg protein): GSH⁺, 17.6; GSH⁻, 0.27; the GSH⁺ reversion, 10.1.

The GSH⁺ reversion was irradiated in the presence of air and in suspension bubbled with argon to remove oxygen. The results of this experiment are shown in Fig. 3. Clearly, the reversion shows a response qualitatively similar to the wild type, and quantitatively, based on its GSH content, to irradiation in reduced oxygen.

The mechanism by which cellular glutathione protects against ionising radiation damage in the presence of oxygen

is not known. A simple question can be dealt with, however. Can growth of the GSH⁻ deficient mutant in the presence of glutathione protect the cells against radiation damage under reduced oxygen tension? The results indicate that this is so; GSH⁻ mutant cells grown in glutathione, washed and resuspended in buffer are consistently more resistant to radiation under reduced oxygen tension: 3,100-fold; 44-fold; 41-fold; 100-fold; 10-fold; 8-fold in six separate experiments. Some variable phenotypic reversal can be achieved.

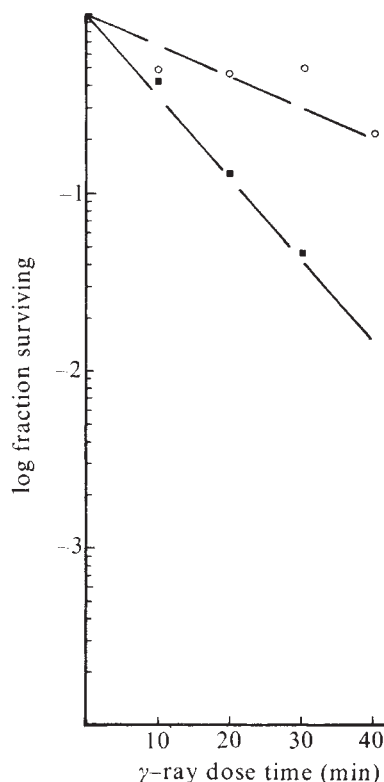
Indirect effects of ionising radiation have been reviewed^{16,17}, and recent summaries of substances that sensitise to radiation presented^{18,19}. Quantitative relationships between radiation response and oxygen tension have been considered linear¹², but this assumption is probably not correct¹¹. Despite these studies, the biochemical basis for the oxygen effect remains obscure.

It has been speculated that the oxygen effect is related to cellular sulphhydryl groups because of the reactive nature of these groups with oxidising radicals produced by irradiation of water. A horizontal study of a number of bacterial strains, attempting to relate radiation resistance to GSH content has been reported²¹. The results presented above limit this view to the oxygen effect. In addition, since total cellular GSH groups are not reduced in GSH⁻ mutants^{13,14}, it suggests specificity to GSH and not sulphhydryl groups *per se*.

The nature of this specificity remains to be determined, and it is not known in what manner GSH participates in cell protection, or at what stage in the radiation process it is needed. Growth in the presence of GSH seems to partially reverse the phenotype of the GSH⁻ mutant, and these experiments need to be expanded as well as a study of the effects of post-radiation addition of GSH.

Methyl glyoxal and diamide [diazene dicarboxylic acid, bis(*N,N*-dimethylamide)] have been shown to reverse the

Fig. 3 Survival of a spontaneous GSH⁺ reversion of the GSH⁻ strain. ■, Bubbled with air; ○, bubbled with argon. Cell growth, preparation and irradiation of cells as in Figs 1 and 2. Reversion to GSH⁺ has restored qualitatively the response to oxygen removal, and almost quantitatively the amount of resistance, since the reversion has only about 50% the GSH content of the wild type. Compare with Fig. 1.



protective effect of anoxia^{18,20}. Since these compounds are reagents reactive with sulphhydryl groups, perhaps specifically with GSH, the mechanism by which they act can now be understood. Presumably, destruction of cellular GSH by these reagents makes cells phenotypically identical to the GSH⁻ mutant.

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Note added in proof: Since the above experiments were conducted, we have received a γ -glutamyl cysteine synthetase mutant, and an independent glutathione synthetase mutant from Apontowei and Berends¹⁴. Both of these strains cannot be protected against ionising radiation damage by oxygen removal.

M. L. MORSE
ROLF H. DAHL

Webb Waring Lung Institute, and
Department of Biophysics and Genetics,
University of Colorado Medical Center,
Denver, Colorado 80262

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- Holthusen, H. *Pflügers Archiv de gesante Physiologie* **181**, 1–25 (1921).
- Kiefer, J. in *Radiation Research: Biomedical, Chemical and Physical Perspectives*, (ed. Nygaard, O. F.) 1025–1037 (Academic, New York, 1975).
- Giles, N. H. & Riley, H. P. *Proc. natn. Acad. Sci. U.S.A.* **36**, 336–344 (1950).
- Baker, W. K. & Sgourakis, E. *Proc. natn. Acad. Sci. U.S.A.* **36**, 176–184 (1950).
- Schwartz, D. *Proc. natn. Acad. Sci. U.S.A.* **38**, 490–494 (1952).
- Hollaender, A., Baker, W. K. & Anderson, E. H. *Cold Spring Harb. Symp. quant. Biol.* **16**, 315–326 (1951).
- Gaulden, M. E. & Nix, M. *Genetics* **35**, 665–666 (1950).
- Hollaender, A. & Stapleton, G. E. *Physiol. Rev.* **33**, 77–84 (1953).
- Burnett, W. T., Stapleton, G. E., Morse, M. L. & Hollaender, A. *Proc. Soc. exp. Biol. Med.* **77**, 636–638 (1951).
- Burnett, W. T., Morse, M. L., Burke, A. W. & Hollaender, A. *J. Bact.* **63**, 591–595 (1952).
- Morse, M. L., Burke, A. W. & Burnett, W. T. *J. Cell. comp. Physiol.* **41**, 407–417 (1953).
- Alper, T. & Howard-Flanders, P. *Nature* **178**, 978–979 (1956).
- Fuchs, J. A. & Warner, H. R. *J. Bact.* **124**, 140–148 (1975).
- Apontowei, P. & Berends, W. *Biochim. biophys. Acta* **399**, 10–22 (1975).
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. *J. biol. Chem.* **193**, 265–275 (1951).
- Bacq, Z. M. *Experientia* **7**, 11–19 (1951).
- Hollaender, A., Stapleton, G. E. & Burnett, W. T. in *Use of Tracers in the Study of Biological Effects of Radiation* 96–113 (CIBA Foundation, London, 1951).
- Ashwood-Smith, M. J., Robinson, D. M., Barnes, J. H. & Bridges, B. A. *Nature* **216**, 137–139 (1967).
- Bridges, B. A. *Adv. radiat. Biol.* **3**, 123–176 (1969).
- Harris, J. W., Koch, C. J., Power, J. A. & Biaglow, J. E. *Radiat. Res.* **70**, 585–596 (1977).
- Bruce, A. K., Sansone, P. A. & MacVittie, T. J. *Radiat. Res.* **38**, 95–108 (1969).

Parallel induction of sister chromatid exchanges and infectious virus from SV40-transformed cells by alkylating agents

ALTHOUGH little is known about the biochemistry of the formation of sister chromatid exchanges (SCEs), it has been suggested^{1–5} that they reflect some aspect of cellular DNA repair. Because agents that promote SCE formation (for example, refs 2, 3 and 6–9) also induce infectious virus from certain SV40-transformed hamster cell lines^{10,11} and because evidence suggests that virus induction involves DNA repair^{12,13}, we have investigated a possible correlation between virus induction and SCE formation. We tested both events in the same cells in identical conditions. Parallel dose-response curves for both SCE formation and induction of infectious SV40 virus were observed with two alkylating agents, mitomycin C and ethyl methane sulphonate (EMS), which required effective concentrations differing by a factor of 10,000.

We used four clones of SV40-transformed hamster kidney cells, differing in their capacity to produce infectious virus¹⁴. Clone A cells and to a lesser extent clone E cells release virus spontaneously. In both cell lines, as well as in clone B cells, which have not been found to produce virus spontaneously, virus induction can be stimulated as much as 10^3 – 10^4 -fold by exogenous agents such as mitomycin C¹¹. Clone G is a non-producing, non-inducible cell line while BHK-21 is an untransformed hamster kidney cell



Fig. 1 Detection of SCEs and their induction by mitomycin C in hamster metaphase chromosomes by BRdU-33258 Hoechst-Giemsa methodology²¹. SV40-transformed hamster kidney cells (clone G) were grown for 24 h in medium (Eagle's minimal essential medium (Gibco) with four times the usual concentration of vitamins and essential amino acids (MEM $\times$ 4), 10% foetal calf serum (Gibco), 2 mM glutamine, penicillin (250 U ml⁻¹, and streptomycin sulphate (250 μ g ml⁻¹) containing 2.5×10^{-5} M BRdU. Cell collection and slide preparation using 33258 Hoechst dye and Giemsa stain were as before^{22,23}, except that: a, a high concentration of colcemid (Calbiochem; final concentration 8 μ g ml⁻¹) was added for the final 6 h as the control; b, mitomycin C (0.03 μ g ml⁻¹) was added 18 h before cell collection.

line which was used as a control. Duplicate samples of cell cultures were grown in the presence of BRdU, exposed to alkylating agents, and, at appropriate times, assayed either for SCE formation or production of infectious virus.

As previously described for other cell types, SCEs were detectable as reciprocal alterations in staining intensity along metaphase chromosomes of hamster cells which had incorporated BRdU for two divisions (Fig. 1a). An average of 11 SCEs was observed per metaphase in uninduced clone G cells. Treatment with mitomycin C (0.03 μ g ml⁻¹) increased the average frequency of SCEs in these cells to 88 per metaphase (Fig. 1b).

The average frequency of SCE formation, normalised per chromosome number of each cell line, increased in all lines in proportion to the dose of either inducing agent (Figs 2a, b). Mitomycin C was effective in inducing SCEs at much lower concentrations than was EMS. For example, a fourfold increase in SCEs over baseline frequency was caused by mitomycin C, in a concentration of less than 20 μ g ml⁻¹ but required EMS at approximately 300 μ g ml⁻¹. Similar frequencies of baseline and induced SCEs were observed for all cell lines. Chromosomal aberrations increased with increasing doses of inducing agent, averaging 3–4% of the number of SCEs. Aberrations were not always detected at the lowest concentrations of inducing agent, whereas SCEs were always measurable and are therefore more sensitive indices of response to the agents.

Virus yields paralleled SCE frequency in their response to increasing doses of mitomycin C or EMS (Figs 2c, d). Absolute levels of virus production were significantly different in clones A, E and B, although the relative responses of the cells to mitomycin C and EMS were similar. Cell survival, as measured by colony-forming ability, was approximately the same for each virus-producing cell line within the concentration ranges used of either agent.

Interclonal differences in absolute virus yields could depend on several factors, such as the nature or sites of viral genome integration, completeness of the integrated genome or mechanisms controlling viral genome excision, replication, transcription or post-transcriptional events. Whatever the explanation, all virus producers showed an induction response which paralleled the SCE increase.

All experiments were performed in the presence of

BRdU, which can induce SCEs^{2,6,8} and certain DNA and RNA viruses in normal and/or transformed cells¹⁴⁻¹⁷. In the hamster clones used in this study BRdU increased the absolute yield of SV40 two to fivefold but had little effect on the dose-dependent response to alkylating agents. The details of BRdU effects on DNA and RNA virus induction are under investigation.

The mechanisms of virus induction and SCE formation may involve DNA repair with some enzymatic features in common. DNA breakage seems to be associated with virus activation from transformed cells containing integrated viral genomes¹¹. Caffeine, which causes the accumulation of gaps in DNA damaged by ultraviolet light in rodent cells, by inhibiting post-replication repair, stimulates the induction of SV40¹³ and SCE¹⁸ by ultraviolet light. It might be the persistence of gaps which results in this enhancement. Pathways of DNA repair, perhaps related to recombinational events and analogous to SOS functions in prokaryotes¹⁹ and possibly in eukaryotes²⁰, may be involved in SCE formation. An association of SCE formation with an error-prone repair process could account for the observed correlation between SCEs, mutagens and carcinogens.

The exact relationship of SCE formation and virus induction remains to be determined, although the two

events show a striking correlation. Further examination of their temporal connection and biochemical characteristics in relation to cellular DNA repair processes may help to explain their biological significance. In any case virus induction is a highly sensitive biological marker for further studies on the mechanism(s) of DNA repair and SCE formation in mammalian cells.

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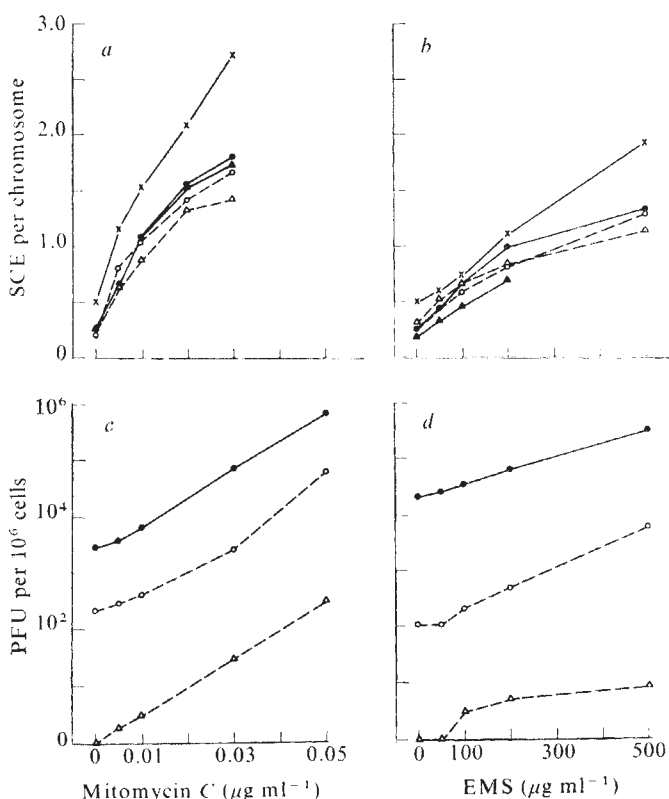
JOAN C. KAPLAN
GLEN B. ZAMANSKY
PAUL H. BLACK

Infectious Disease Unit,
Massachusetts General Hospital,
Boston, Massachusetts, 02114 and
Departments of Microbiology and Molecular
Genetics, and Medicine,
Harvard Medical School,
Boston, Massachusetts, 02115

SAMUEL A. LATT

Clinical Genetics Division,
Mental Retardation Center,
Children's Hospital Medical Center,
Boston, Massachusetts, 02115

Fig. 2 Induction of SCEs and infectious virus in different clones of SV40-transformed hamster kidney cells by mitomycin C or EMS. Cells were cultured for two divisions in growth medium containing 2.5×10^{-6} M BRdU. Eighteen hours before collection for SCEs, either mitomycin C or EMS was added to the culture medium. Cell collection and slide preparation were as described for Fig. 1. SCE frequencies are expressed per chromosome (a and b). ●, Clone A; ○, E; △, B; ▲, G; X, untransformed BHK-21 hamster cells. Replicate cultures for assay of infectious virus production were treated as above except that mitomycin C or EMS was removed 24 h after addition to the cultures. Cells were washed with phosphate-buffered saline and fresh growth medium was added before the cultures were reincubated at 37 °C for 72 h. Procedures for collection, preparation of cell-free extracts and plaque assay for SV40 virus infectivity were as described¹¹. Virus yields are expressed as plaque-forming units (PFU) per 10^6 cells present at the time of addition of the inducing agent (c and d). ●, Clones A; ○, E; △, B.



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1. Kato, H. *Expl Cell Res.* **82**, 383-390 (1973).
2. Latt, S. A. *Proc. natn. Acad. Sci. U.S.A.* **71**, 3162-3166 (1974).
3. Perry, P. & Evans, H. J. *Nature* **258**, 121-124 (1975).
4. Latt, S. A., Stetten, G., Juergens, L. A., Buchanan, G. R. & Gerald, P. S. *Proc. natn. Acad. Sci. U.S.A.* **72**, 4066-4070 (1975).
5. Wolff, S., Rodin, B. & Cleaver, J. E. *Nature* **265**, 347-349 (1977).
6. Wolff, S. & Perry, P. *Chromosoma* **48**, 341-353 (1974).
7. Kato, H. *Nature* **249**, 552-553 (1974).
8. Kato, H. *Nature* **251**, 70-72 (1974).
9. Wolff, S. A. *Rev. Genet.* **11**, 183-201 (1977).
10. Rothschild, H. & Black, P. H. *Virology* **42**, 251-256 (1970).
11. Kaplan, J. C., Wilbert, S. M., Collins, J. J., Rakusanova, T., Zamansky, G. B. & Black, P. H. *Virology* **68**, 200-214 (1975).
12. Kaplan, J. C., Kleinman, L. F. & Black, P. H. *Virology* **68**, 215-220 (1975).
13. Zamansky, G. B., Kleinman, L. F., Little, J. B., Black, P. H. & Kaplan, J. C. *Virology* **73**, 468-475 (1976).
14. Fogel, M. *Nature new Biol.* **241**, 182-184 (1973).
15. Hampar, B., Derge, J. G., Martos, L. M. & Walker, J. L. *Proc. natn. Acad. Sci. U.S.A.* **69**, 78-82 (1972).
16. Lowy, D. R., Rowe, W. P., Teich, N. & Hartley, J. W. *Science* **174**, 155-156 (1971).
17. Aaronson, S. A., Todaro, G. J. & Scolnick, E. M. *Science* **174**, 157-159 (1971).
18. Sasaki, M. S. *Nature* **269**, 623-625 (1977).
19. Witkin, E. M. *Bact. Rev.* **40**, 869-907 (1976).
20. Troll, W., Meyn, S. & Rossman, T. G. *Gatlinburg Symp. Mechanisms of Tumor Promotion and Cocarcinogenesis*, (in the press).
21. Perry, P. & Wolff, S. *Proc. natn. Acad. Sci. U.S.A.* **70**, 3395-3399 (1973).
22. Latt, S. A. *Proc. natn. Acad. Sci. U.S.A.* **70**, 3395-3399 (1973).
23. Latt, S. A., Allen, J. W., Rogers, W. E. & Juergens, L. A. in *Handbook of Mutagenicity Test Procedures* (eds Kilbey, B., Legator, M. & Ramel, C.) 275-291 (Elsevier-North Holland, New York, Amsterdam, 1977).

Induction of autoreactive T lymphocytes and their suppressor cells by cyclophosphamide

THERE is increasing evidence of potentially autoreactive cells in normal animals^{1,2}. Expression of autoimmunity, however, may not arise as a primary effector cell abnormality but more possibly as the consequence of a defect in a thymus-dependent control mechanism³⁻⁵. Cyclophosphamide (CY) enhances the capacity of mice to give T cell-dependent responses, such as delayed type hypersensitivity (DTH) induced by sheep red blood cells⁶. It was suggested that B cells were the targets of CY action⁷ and that the enhanced DTH reaction was due to inhibition of antibody production and consequently to lack of the antigen-antibody complexes responsible for inhibition of DTH. Askenase *et al.*⁸ presented evidence for an alternative explanation for enhancement of DTH by CY. They demonstrated augmented DTH to sheep red blood cells with doses of CY which did not influence antibody responses, suggesting the existence of CY-sensitive suppressor cells. Similar conclusions

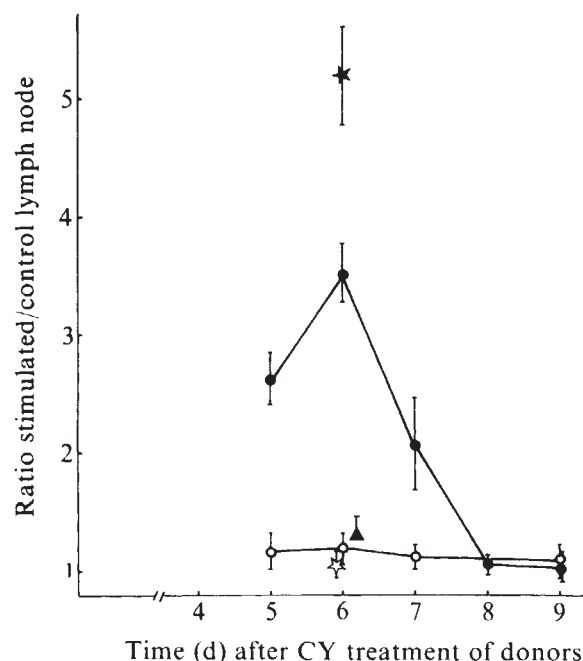


Fig. 1 Local GvH reaction in syngeneic host. Female BALB/c or AKR mice, 6–8-weeks old, were injected intraperitoneally with 125 mg kg⁻¹ cyclophosphamide (CY). Spleen cells were collected at various intervals. Cells from 6–12 spleens were pooled, suspended in Hanks BSS (supplemented with 5% foetal calf serum, 100 units ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin) and passed through a nylon wool column¹⁰. The eluted cells were washed three times with serum-free medium. As tested on cell smears stained with either Giemsa or with fluorescein-conjugated rabbit anti-mouse immunoglobulin, the nylon wool filtered cells were depleted on both phagocytic cells (less than 0.1%) as well as on B lymphocytes (less than 5%). In some experiments, nylon wool-filtered AKR spleen cells were incubated with anti-theta serum (C3H anti-AKR Thy-1.1 from Searle Diagnostic, High Wycombe, England) and guinea pig complement. Control cells were incubated with normal C3H serum and complement. After incubation, the cells were washed three times with serum-free medium. 3 × 10⁶ cells in a volume of 0.05 ml were injected subcutaneously into the right hind footpad of a syngeneic recipient. Six days later, the draining and the contralateral lymph nodes were removed and weighed. The results are expressed as the ratio of the weights of stimulated to contralateral unstimulated popliteal lymph node. ●, Cells derived from CY-treated BALB/c donors. ○, Cells derived from untreated BALB/c controls. ★, Cells derived from CY-treated AKR donors and injected into AKR recipients. ☆, Cells derived from untreated AKR controls. ▲, Cells derived from CY-treated AKR donors after anti-theta treatment. Each value represents the mean ± s.e.m. obtained from 5–7 recipients.

have been drawn by Röellinghoff *et al.*⁹, who observed induction of cytotoxic lymphocytes in mice to hapten-conjugated syngeneic cells after treatment with CY. We report here that injection of normal mice with CY results in manifestation of autoreactive T lymphocytes followed by the appearance of suppressor cells counteracting autoreactivity.

Balb/c or AKR mice were injected with a single dose of CY (125 mg kg⁻¹). At various time intervals splenic T cells (prepared by passing the cells through nylon-wool column¹⁰) were injected subcutaneously into the footpads of syngeneic recipients, and the resulting local graft versus host (GvH) reaction^{2,11} was determined in the draining lymph nodes. Autoreactive cells could be detected in the spleens of donors with a peak 6 d after CY treatment (Fig. 1). Autoreactive cells treated with anti-theta serum and complement failed to induce a GvH reaction. As in local GvH reactions induced by allogeneic cells^{12,13}, the GvH reaction in our syngeneic system depends on cells of host origin. This was shown by failure of X-irradiated (800 r) or CY-treated (125 mg kg⁻¹) recipients to give a GvH reaction when injected with autoreactive cells 1 d after this procedure. T cells derived from donors 8–9 d after CY treatment did not elicit a GvH reaction. Moreover, these cells, mixed with autoreactive cells, abolished the GvH reaction (Table 1), but if the donors were intravenously injected 20 h after CY with 5 × 10⁷ syngeneic thymocytes autoreactive cells were not detectable. Splenic T cells of these animals collected 5 d after thymocyte injection suppressed the activity of autoreactive cells (data not shown), indicating CY-sensitivity of suppressor cell precursors.

It has been shown that T cells of neonatally⁴ or adult⁵ thymectomised mice injected into syngeneic recipients induce GvH reactions. The authors suggest that this phenomenon is due either to impaired thymus functions or loss of T-suppressor cells. This implies the coexistence of committed autoreactive and suppressor cells in normal spleen cell populations. Because suppressor cells are CY-sensitive, our results could be interpreted simply as being due to loss of suppressor cells, permitting detection of autoreactive cells; but the findings that normal spleen cells failed to inhibit expression of autoreactivity (Table 1) and that autoreactive cells do induce GvH reactions in normal syngeneic mice, argue against pre-existence of already committed suppressor cells. One of the crucial questions concerns the nature of self antigens which are recognised by the cells involved in this autoimmune phenomenon. Do autoreactive lymphocytes recognise normal or altered self-components, and what is the nature of the alteration? As previously shown¹¹, mice injected with 125 mg kg⁻¹ CY are depleted (1–4 d after this treatment) of B cells and of subsets of T cells. The damage to the cells in the spleen is followed by a burst of rapidly proliferating B cells. B cell mitogens have been

Table 1 Demonstration of autoreactive lymphocytes and their suppression by regulatory cells

Treatment of donors	Nylon wool	No. of cells transferred	No. of recipients	Local GvH response lymph node ratio	
				Weight ± s.e.m.	Cell number
none	no	3 × 10 ⁶	10	1.18 ± 0.08	1.28
none	yes	3 × 10 ⁶	16	1.16 ± 0.09	1.14
a	no	6 × 10 ⁶	6	3.01 ± 0.32	3.80
	yes	3 × 10 ⁶	20	3.23 ± 0.21	4.43
b	yes	3 × 10 ⁶	11	1.05 ± 0.08	1.10
c	yes	3 × 10 ⁶	5	1.33 ± 0.37	1.69
d cells from a	yes	3 × 10 ⁶			
+ normal spleen cells	yes	3 × 10 ⁶	6	3.11 ± 0.30	4.09
d cells from a	yes	3 × 10 ⁶			
+ cells from b	yes	1.5 × 10 ⁶	6	1.38 ± 0.15	1.45
d cells from a	yes	3 × 10 ⁶			
+ cells from b	yes	3 × 10 ⁶	6	1.22 ± 0.11	1.07

Spleen cells were obtained from BALB/c donors as described in Fig. 1, for a, 6 d and b, 8 d after CY treatment; c, CY-treated donors were intravenously injected 20 h later with 5 × 10⁷ normal thymocytes, spleens were removed 6 d after CY d, cells were mixed and injected in a volume of 0.05 ml into the hind footpad of the recipients. The results of the local GvH response are either calculated as the ratio in the weights ± s.e.m. or as the ratio in the cell content between the stimulated and contralateral unstimulated lymph nodes 6 d after cell transfer. The ratios for the number of cells were obtained from pooled lymph nodes in each experimental group.

shown^{15,16} to induce the appearance of endogenous C-type RNA viruses in spleen cell culture supernatants and at the surface of splenic B cells. Therefore, it is possible that the 'autoreactive' T cells may recognise altered self antigens (possibly virus-altered) on proliferating B cells. This is supported by the findings that AKR mice, known to contain genetically much higher titres of endogenous ecotropic and xenotropic viruses¹⁷, when injected with CY, develop a greatly increased number of autoreactive cells, as compared to BALB/c mice.

The results indicate that CY treatment induces the appearance of 'new' antigenic sites (perhaps present at the surface of primitive B cells or as altered self-components due to endogenous virus induction) and the transient depletion of the spleen on T-suppressor cells. Both effects acting together permit manifestation of 'autoreactive' cells in normal animals.

JOHANNA L'AGE-STEHR
TIBOR DIAMANTSTEIN

Immunology Research Unit,
Klinikum Steglitz,
Free University Berlin,
1000 Berlin 45, Germany

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1. Cohen, J. R., Globerson, A. & Feldmann, M. J. *J. exp. Med.* **133**, 834-845 (1971).
2. Cohen, J. R. & Weckerle, H. J. *exp. Med.* **137**, 224-238 (1973).
3. Talal, N. *Prog. clin. Immun.* **2**, 101-120 (1974).
4. Small, M. & Trainin, N. *Cell Immun.* **20**, 1-11 (1975).
5. Carnaud, C., Charreire, J. & Bach, J. F. *Cell Immun.* **28**, 274-283 (1977).
6. Lagrange, P. H., Mackaness, G. B. & Miller, T. E. *J. exp. Med.* **139**, 1529-1539 (1974).
7. Turk, J. L. & Poulter, L. W. *Clin. exp. Immun.* **10**, 285-296 (1972).
8. Askenase, P. W., Hayden, B. J. & Gershon, R. K. *J. exp. Med.* **141**, 697-702 (1975).
9. Röellinghoff, M., Starzinski-Powitz, A., Pfizenmayer, K. & Wagner, H. *J. exp. Med.* **145**, 455-459 (1977).
10. Julius, M. H., Simpson, E. & Herzenberg, L. A. *Eur. J. Immun.* **3**, 643-645 (1973).
11. Ford, W. L., Burr, W. & Simonsen, N. *Transplantation* **10**, 258-266 (1970).
12. Bonney, W. W. & Feldbush, T. L. *Transplantation* **15**, 215-220 (1973).
13. Rolstad, B. *Transplantation* **21**, 117-123 (1976).
14. L'age-Stehr, J., Blistein-Willinger, E. & Diamantstein, T. *Z. Immun. Forsch. exp. Ther.* **153**, 327 (1977).
15. Moroni, C., Schumann, G., Robert-Guroff, M., Suter, E. & Martin, D. *Proc. natn. Acad. Sci. U.S.A.* **72**, 535-538 (1975).
16. Phillips, S. M., Stephenson, J. R. & Aaronson, S. A. *J. Immun.* **118**, 662-666 (1977).
17. Hartley, J. W., Rowe, W. P., Capps, W. J. & Huebner, R. J. *J. Virol.* **3**, 126-132 (1969).

was employed in the present studies. The fractionated SF samples were stored in aliquots at -20°C . Serum fractions from normal individuals were used after being stored for two days and fractions from athymic patients were used after two weeks' storage. As reported by Astaldi *et al.*², we observed that the serum fraction retained by the filter (MW $> 50,000$) was inactive in the assay used.

SF samples were evaluated for their ability to increase cyclic AMP levels of mouse thymocytes. Incubations were terminated either by rapid freezing and assayed for cyclic AMP *in situ* according to the procedure of Astaldi *et al.* ('unpurified' sample), or terminated by trichloroacetic acid precipitation followed by ether extraction and Dowex-1 column purification before assaying for cyclic AMP (ref. 3) ('purified' sample). Because the SF samples contained cyclic AMP (up to 50% of the total), the cyclic AMP levels of the serum were subtracted from the total cyclic AMP measured in the samples containing both cells and the factor, thus giving the net value provided by the cells. Basal cellular levels of cyclic AMP in the unpurified samples were 1.7 pmol per 20×10^6 cells and 2.8 pmol per 20×10^6 cells in purified samples (Table 1). Total cyclic AMP levels were increased in a dose-dependent manner by both normal and athymic SF, but when the cyclic AMP component of the SF was subtracted, cellular increases were marginal and inconsistent in samples assayed by the procedure of Astaldi *et al.*². In purified samples, however, both normal and athymic SF's stimulated cyclic AMP levels in a dose-dependent fashion by up to two and a half times. These increases were small in comparison with the stimulatory effect of isoproterenol (Table 1 and refs 2, 4).

Manipulation of the thymocytes or of the SF's did not change their effects on cyclic AMP levels. Thus, serum fractions prepared from normal donors by rapid defibrination of whole blood followed by immediate transfer to an ice bath at 4°C produced no greater enhancement of cyclic AMP levels in thymocytes than those prepared by allowing whole blood to

Table 1 Effects of athymic and normal serum fractions on mouse thymocyte cyclic AMP levels

Addition		Unpurified sample		Purified sample	
		Total cyclic AMP	Net cyclic AMP	Total cyclic AMP	Net cyclic AMP
Normal SF	0	1.70	1.70	2.84	2.84
	0.125 ml	2.26	1.50	4.21	3.17
	0.25 ml	3.73	2.20	7.21	5.12
	0.50 ml	4.70	1.64	10.99	6.83
	0.25 ml	1.53	0	2.08	0
Athymic SF	0	1.70	1.70	2.84	2.84
	0.125 ml	2.96	2.28	4.89	3.97
	0.25 ml	3.56	2.20	6.21	4.36
	0.50 ml	4.99	2.27	10.36	6.66
	0.25 ml	1.36	0	1.85	0
10 ⁻⁵ M Isoproterenol		+		185.6	185.6

Serum fractions of less than 50,000 MW were prepared from two normal and two athymic donors as described by Astaldi *et al.*². Thymocytes were obtained from 6-10-week female C57BL/6 mice and resuspended in triplicate in Hank's Balanced Salt Solution without phenol red. Each tube contained 20×10^6 cells. 0.125, 0.25 or 0.5 ml aliquots of the serum fractions were added to make a total volume of 1 ml per tube. After incubating for 5 min at 37°C , the incubation was either terminated by rapid freezing and assayed for cyclic AMP *in situ* as previously described by Astaldi *et al.*², or terminated by trichloroacetic acid precipitation followed by ether extraction and Dowex-1 column purification before assaying for cyclic AMP (ref. 3). Aliquots of purified samples were tested with a phosphodiesterase preparation. $>95\%$ of the cyclic AMP measured was destroyed by this treatment, confirming the purity of the material. Results are expressed as pmol cyclic AMP measured per 20×10^6 cells. 'Total cyclic AMP' is the total amount of cyclic AMP measured, 'Net cyclic AMP' has the cyclic AMP content of the SF subtracted out, so that the values reflect only cell-produced cyclic AMP.

Is there a circulating human thymic factor that induces cyclic AMP synthesis?

THE action of factors which have been extracted from the thymus or found circulating in peripheral blood and which are active in *in vitro* lymphocyte differentiation assays¹ has been extensively studied. Astaldi *et al.*² have described a factor (SF) of molecular weight (MW) less than 50,000 present in the serum of normal donors; a thymic site of production was suggested because it was not found in serum from athymic donors. It also significantly elevated cyclic AMP levels in mouse thymocytes. Because of the potential significance of this finding to the study of thymic factor action, we have attempted to corroborate these findings but, as reported here, we have observed that a serum fraction from both normal and athymic individuals can raise cyclic AMP levels in mouse thymocytes. Neither serum fraction, however, produced the degree of stimulation of cyclic AMP levels described by Astaldi *et al.*². Increases in cyclic AMP were much lower than those observed using the β -adrenergic agonist isoproterenol. We are therefore unable to confirm the existence of a circulating thymic factor in humans which raises cyclic AMP levels in mouse thymocytes.

Sera were initially obtained from two normal donors and two myasthenia gravis patients who had undergone thymectomy (samples by kind permission of Dr J. Posner, Memorial Hospital and Dr G. Jenkins, Mt Sinai Hospital, New York City). As described by Astaldi *et al.*², we separated the fresh serum into two fractions using Amicon CF50 filters. The fraction passing through the filter containing the SF (MW $< 50,000$)

clot. Moreover, neither basal nor stimulated levels of cyclic AMP were affected by pretreating the thymocytes on nylon wool columns.

Table 1 shows that both total and net cellular cyclic AMP levels (basal and stimulated) are much higher after sample purification, suggesting that there may be factors in the unpurified material which interfere with the cyclic AMP assay. We have previously found that such factors must be removed by Dowex-1 chromatography before accurate cyclic nucleotide measurements can be made³. Phosphodiesterase treatment confirmed that our purified samples after Dowex chromatography were >95% cyclic AMP. It is possible that Astaldi *et al.* were not measuring true cyclic AMP levels, but rather some other as yet unknown contaminating material. All our subsequent data were obtained by isolating and purifying cyclic AMP from the sample after acid precipitation.

Our results differ from those of Astaldi *et al.*² in that their basal levels of cyclic AMP were higher than ours (10 compared to our 2.8 pmol per 20×10^6 cells). Our values are in close agreement with those obtained by others^{4,5}. Moreover, although we have also observed that stimulation with SF is dose-dependent, we have observed much lower stimulated levels and smaller net increase in cyclic AMP than did Astaldi *et al.* The reason for these discrepancies remains unknown. Differences may in part relate to the relative health of the cells employed, failure to calculate cyclic AMP levels in the SF sample and to subtract it from the total, and possible difficulties in measuring cyclic AMP in unpurified samples.

Our preliminary evidence also indicated that both normal and athymic serum fractions exhibited almost identical dose-dependent elevations of cyclic AMP levels in contrast to the findings of Astaldi *et al.*² whose serum fractions from athymic patients were without effect on cyclic AMP levels in thymocytes. We therefore prepared SF's from a further seven patients who had undergone thymectomy following treatment for myasthenia gravis (age range 26–54 yr; time post-thymectomy 3 months to 12 years 3 months). The effect on mouse thymocytes of the serum fractions from eight of the nine athymic patients is shown in Table 2, and compared with the effects of identical fractions prepared from three normal donors.

SF's were assayed either the day they were prepared or after being stored for 24 h at -20°C . Samples were stored in aliquots at -20°C and retested at intervals over a period of up to two months. Although variations in cyclic AMP elevation were observed over this period with individual SF's, there was no correlation between the level of cyclic AMP induced and sample storage time. It is apparent from Table 2 that the levels of cyclic AMP induced by serum fractions from athymic patients are almost identical to those induced by fractions from normal donors. SF from only one athymic patient failed to induce increases in cyclic AMP. Although the treatment of this patient was not appreciably different from that of the eight others, her clinical state was one of the most serious despite thymectomy. This suggests that lack of a thymus may not be central and that the patient may be deficient in some other circulating endocrine product which could raise cyclic AMP levels.

Table 2 Effects of serum fractions prepared from normal and athymic human donors on cyclic AMP levels in mouse thymocytes

Serum fraction volume	Normal	Athymic
0	1.00	1.00
0.125 ml	1.61 $\pm$ 0.27 (9)	1.76 $\pm$ 0.13 (12)
0.25 ml	2.16 $\pm$ 0.35 (9)	2.93 $\pm$ 0.29 (10)
0.50 ml	4.30 $\pm$ 2.04 (6)	4.66 $\pm$ 1.05 (5)

Results are expressed as stimulation with regard to control $\pm$ s.e.m. (no. of separate determinations). Cyclic AMP present in each serum volume was subtracted from total cyclic AMP measured. Experimental conditions as in legend to Table 1. Basal levels of cyclic AMP, 3.6 ± 0.4 pmol per 20×10^6 cells.

Our observations are at variance with the findings of Astaldi *et al.*², who reported that serum fractions from 18 out of 18 athymic donors could not elevate cyclic AMP levels in mouse thymocytes. We suggest that a cyclic AMP-raising material is present in the serum and that the severity of illness rather than the absence of a thymus may determine whether the factor is present in serum. In any case, we consider it more probable that the factor is synthesised outside the thymus, and is in no way related to circulating thymic factors⁶ the activity of which has been shown to decrease with time after thymectomy in experimental animals. Several circulating products, such as prostaglandins or adrenaline, with known cyclic AMP-raising properties in lymphocytes, may be likely candidates and defects in the secretion of one of these substances may account for the single athymic donor not showing cyclic AMP-raising activity in her serum.

In summary, we could find no evidence for a serum substance dependent on the presence of a thymus and acting to stimulate cyclic AMP synthesis in mouse thymocytes. We suggest that the rise in cyclic AMP in mouse thymocytes is due to a non-thymic, circulating material.

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G. H. SUNSHINE
R. G. COFFEY
J. W. HADDEN

Sloan Kettering Institute,
New York, New York 10021

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1. Friedman, H. (ed.) *Ann. N.Y. Acad. Sci.* **249** (1975).
2. Astaldi, A., Astaldi, G. C. B., Schellekens, P. Th. A. & Eijssvoegel, V. P. *Nature* **260**, 713–715 (1976).
3. Hadden, J. W., Hadden, E. M., Sadlik, J. R. & Coffey, R. G. *Proc. natn. Acad. Sci. U.S.A.* **73**, 1717–1721 (1976).
4. Bach, M. A. *J. clin. Invest.* **55**, 1074–1081 (1975).
5. Oppenheim, J. J., Schneyour, A. & Kook, A. I. *J. Immun.* **116**, 1466–1472 (1976).
6. Bach, J. F., Dardenne, M., Pleau, J. M. & Bach, M. A. *Ann. N.Y. Acad. Sci.* **249**, 186–210 (1975).

A. ASTALDI, G. C. B. ASTALDI, P. TH. A. SCHELLELKENS AND V. P. EIJSSVOEGEL REPLY—To date, we have investigated sera from 56 thymectomised myasthenic patients (mean age 32.1 yr, s.e.m. $\pm$ 1.8, time post-thymectomy 1 month to 19 yr), 37 non-thymectomised myasthenic patients (mean age 44.8 yr, s.e.m. $\pm$ 3.4) and 87 normal healthy individuals (mean age 29.0 yr, s.e.m. $\pm$ 1.2). The SF activity was very low in the thymectomised donors (mean 15.6 pmol cyclic AMP per 2×10^7 cells, s.e.m. $\pm$ 3.1), but consistently demonstrable both in normal healthy individuals (mean 76.9 pmol cyclic AMP per 2×10^7 cells, s.e.m. $\pm$ 4.0) and in non-thymectomised myasthenic patients (mean 56.0 pmol cyclic AMP per 2×10^7 cells, s.e.m. $\pm$ 7.4). The difference in SF activity between normals and non-thymectomised myasthenic patients is probably due to age, because we observe that the level of SF declines progressively after the age of 30. Interestingly, within the group of the thymectomised donors two populations could be distinguished, a major one ($n = 43$) with almost no SF activity (mean 6.0 pmol cyclic AMP per 2×10^7 cells, s.e.m. $\pm$ 1.1) and a minor one ($n = 13$) with SF activity (mean 47.0 pmol cyclic AMP per 2×10^7 cells, s.e.m. $\pm$ 7.6) which is closer to that in control groups. This finding is not unexpected in view of the well known 20% incidence of ectopic thymus in humans^{1,2}; also, in some patients the thymectomy may have been incomplete. Repeated determinations of SF activity before and after thymectomy revealed that the SF activity in humans decreases dramatically after thymectomy (Fig. 1).

Sunshine *et al.* state that the cyclic AMP present in the SF should be subtracted from cyclic AMP measured in the samples after incubation. The assay method that we use, however, (see legend to Fig. 1) is rather different from that of Sunshine *et al.* Contrary to their assay, we are measuring only the net cellular cyclic AMP, for by washing the cells before the extraction of cyclic AMP, any cyclic AMP present outside the cells is removed. This could be further sustained by the finding

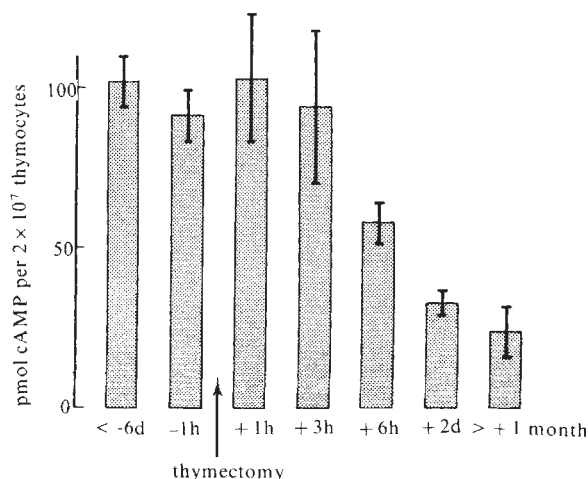


Fig. 1 SF activity at different time intervals before and after thymectomy (mean $\pm$ s.e.m. of seven myasthenic patients). Human blood was collected and immediately defibrinated by shaking for 8–10 min with glass beads. The defibrinated blood was immediately transferred to a plastic tube kept in melting ice and centrifuged at $+4^\circ\text{C}$ for 15 min at 600g in order to obtain serum. Seven ml of serum was immediately filtered through an Amicon Centrifo CF 50 A filter (MW cut-off 50,000). Filters were centrifuged at $+4^\circ\text{C}$ for 1 h at 600g. The ultrafiltrate (SF) was divided into appropriate aliquots, frozen at -100°C and lyophilised. All samples were processed in the same way and tested within a month. We demonstrated that, once lyophilised, samples retain SF activity for a period of over 8 months. Cyclic AMP-free and prostaglandin-free SF was obtained by means of Bio-Rad AG1-X8 chromatography. SF was eluted with 0.1 N formic acid and relyophilised. As control we used SF diluted with 0.1 N formic acid and relyophilised without chromatography. Lyophilised material was reconstituted, just before assay, to the original volume of the filtrate with distilled water. Thymocytes were obtained from C57BL/6 mice aged 6–8 weeks by means of organ dissociation through a metal sieve and subsequent filtration through nylon wool columns. Each assay tube contained 2.5 ml SF, 2.5 ml Earle's solution and 10^7 thymocytes. After 5 min of incubation at 37°C , the tubes containing the cell suspension were centrifuged at $+4^\circ\text{C}$ for 5 min at 600g. Cell viability, both before and after incubation, was $>95\%$. The supernatant was discarded and the pellet resuspended in 0.5 ml of 50 mM Tris-HCl, 4 mM EDTA buffer, pH 7.5, by means of a vortex mixer. Tubes were placed in liquid nitrogen for 1 min and subsequently in a boiling water bath for 2–3 min. Coagulated proteins were spun down at $+4^\circ\text{C}$ for 5 min at 1,500g. The supernatant was directly assayed for cyclic AMP as described⁵ or further purified by means of Bio-Rad AG1-X8 chromatography. Cyclic AMP was eluted with 2N formic acid according to the method of Kuehl *et al.*³. Results were expressed in picomoles of cyclic AMP per 2×10^7 cells. Values reported here were obtained by subtracting the background cellular levels of cyclic AMP (that is, the level of cyclic AMP in thymocytes incubating as such).

that cyclic AMP-free SF (after Bio-Rad AG1-X8 chromatography) had activity similar to SF prepared without chromatography.

Sunshine *et al.* suggested that we might be measuring material other than cyclic AMP, but phosphodiesterase treatment of extracted cellular cyclic AMP confirmed that the material measured was $>90\%$ cyclic AMP. The presence of inhibitory factors is very unlikely because, when a known amount of reference cyclic AMP was added to the extracted cellular cyclic AMP, we recovered $>95\%$ of the expected value, again indicating the correctness of our cyclic AMP measurements. We have purified the extracted cellular cyclic AMP by means of ion exchange chromatography as reported by Kuehl *et al.*³ without any effect on the significance of our findings. In addition, the values of cyclic AMP that we measured in unpurified samples essentially agree with those observed by others⁴, both for isoproterenol and prostaglandin E_1 -stimulated lymphoid cells.

A further discrepancy between the results of Sunshine *et al.* and ourselves is the great difference in activity that we observed between SF prepared by rapid defibrination of fresh blood and SF obtained from blood just allowed to clot; when blood from

normal donors was allowed to clot at room temperature for 1 h, we could find almost no SF activity (mean 6.2 pmol cyclic AMP per 2×10^7 cells, s.e.m. ± 1.2 , $n = 9$). This value is similar to those reported by Sunshine *et al.*, which we regard as very low. It should be stressed that active SF preparations can only be obtained if the extraction procedure described by us is exactly followed, including rapid defibrination.

Sunshine *et al.* raised the possibility that the SF activity might be due to adrenaline or prostaglandins. This is very unlikely in view of the target cell specificity of SF as compared to the β -adrenergic stimulator isoproterenol and to prostaglandin E_1 ⁵. Hadden *et al.*⁶ demonstrated the presence of adrenergic receptors in human lymphocytes and Smith *et al.*⁷ showed that prostaglandins and β -adrenergic stimulators increase cellular cyclic AMP in human peripheral blood lymphocytes, while α -adrenergic stimulators do not. According to this view, adrenaline should stimulate cyclic AMP by means of interaction with the β -adrenergic receptors. We found⁵ that SF does not increase cyclic AMP levels in human peripheral blood lymphocytes. This rules out the possibility that the SF activity might be due to adrenaline or prostaglandins. We also reported⁵ that the β -adrenergic inhibitor propranolol does not to any extent inhibit the SF activity, indicating that SF is not acting on the β -adrenergic receptors.

We have demonstrated⁸ that the target cells for SF are among the hydrocortisone (HC)-sensitive thymocytes and that SF induced HC resistance, a maturation step reported to be induced also by a thymic extract⁹ and by a circulating thymic factor¹⁰. When mouse thymus was depleted of HC-sensitive cells by means of HC treatment *in vivo*, we found that cells recovered (HC-resistant) could not be stimulated to increase their cyclic AMP level by SF. On the contrary, prostaglandin E_1 induced HC-resistant cells to increase cyclic AMP at a level similar to that found when the total thymic population was used as prostaglandin target, again indicating that SF is not acting on the prostaglandin receptor. This could be further supported by the finding that prostaglandin-free SF (after Bio-Rad AG1-X8 chromatography) had activity similar to SF prepared without chromatography.

Finally, we found (Schellekens *et al.*, in preparation) that SF activity was almost absent in seven children with thymic-dependent immunodeficiencies and present in four children with severe combined immunodeficiency disease. Three children, almost completely lacking in SF activity, were treated with the partially purified thymic extract, thymosin fraction V, prepared by Hoffmann-La Roche according to Hooper *et al.*¹¹ Shortly after initiation of thymosin treatment, we observed¹² the appearance in the patients' blood of SF or SF-like cyclic AMP increasing activity. This activity disappeared after thymosin was stopped in one patient. Since thymosin does not induce cyclic AMP increase when tested directly on mouse thymocytes *in vitro*¹³, the SF (-like) activity found during thymosin treatment might be due to *in vivo* activation either of or by thymosin.

The only remaining possible explanation for the failure of Sunshine *et al.* to reproduce our findings seems to be the difference in technique. We realise that the description of methodology previously reported⁵ could be misinterpreted. In fact, as stated above, non-appropriate handling of the blood may cause loss of SF activity. When tested, such SF will give a very modest cyclic AMP stimulation in the range of values reported by us for thymectomised donors (ref. 5 and above), which is of exactly the same order of magnitude as that reported by Sunshine *et al.* both for normals and thymectomised individuals.

A. ASTALDI
GIULIA C. B. ASTALDI
P. TH. A. SCHELLEKENS
V. P. ELSVOOGEL

Central Laboratory of the Netherlands Red Cross Blood
Transfusion Service and the Laboratory of Experimental and
Clinical Immunology of the University of Amsterdam,
PO Box 9190, Amsterdam, The Netherlands

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1. Rieffel, H. & Le Mee, J. C. *r. hebdom. Seanc. Acad. Sci., Paris* **148**, 519–520 (1909).
2. Gilmour, J. R. *J. Pathol. Bacteriol.* **52**, 213–218 (1941).
3. Kuehl, F. A. Jr *et al. Proc. natn. Acad. Sci. U.S.A.* **71**, 1866–1870 (1974).
4. Bach, M. A. *J. clin. Invest.* **55**, 1074–1081 (1975).
5. Astaldi, A. *et al. Nature* **260**, 713–715 (1976).
6. Hadden, J. W., Hadden, E. M. & Middleton, E. Jr *Cell. Immun.* **1**, 583–595 (1970).
7. Smith, J. W. *et al. J. clin. Invest.* **50**, 432–441 (1971).
8. Astaldi, G. C. B. *et al. Eur. J. Immun.* **7**, 836–840 (1977).
9. Trainin, N., Levo, Y. & Rotter, V. *Eur. J. Immun.* **4**, 634–637 (1974).
10. Bach, J. F. *et al. Transplantation Proc.* **7**, 25–30 (1975).
11. Hooper, J. A. *et al. Ann. N.Y. Acad. Sci.* **249**, 125–144 (1975).
12. Astaldi, A. *et al. J. Immun.* **119**, 1106–1108 (1977).
13. Naylor, P. H. *et al. Biochem. biophys. Res. Commun.* **73**, 843–845 (1976).

Fibroblast-conditioned medium contains cell surface proteins required for cell attachment and spreading

PROLIFERATION in diploid fibroblasts is limited to cells that are attached and spread on a substrate. The attachment and spreading activities of normal cells are enhanced in the presence of media conditioned by the growth of diploid fibroblasts^{1,2}, and a cell surface protein (LETS/CSP)³ extracted from normal cells enhances the attachment of transformed cells^{4,5}. We report here that the activities present in conditioned medium are due to several high molecular weight glycoproteins ('released proteins'). In addition, we find that the same activities can be extracted ('extracted proteins') from the surfaces of whole cells. The results establish that growth related biological activities are transferred intercellularly through the release and absorption of cell surface components.

A comparison of released and extracted proteins is shown in Fig. 1. Figure 1a and e show that approximately 90% of the Coomassie blue staining material was located in three major bands of >150,000 apparent molecular weight (MW). The rest of the material migrated between 35,000 and 150,000 MW and was removed in the supernatant fraction after treatment with a 35% saturated solution of ammonium sulphate. The three released proteins were clearly the products of cellular synthesis. Metabolic incorporation of either ¹⁴C-leucine (Fig. 1f–h) or ¹⁴C-glucosamine (Fig. 1j) labelled all major bands. The labelling patterns of the extracted proteins (Fig. 1i and k) were similar. The band in the middle of the gels, stained with Coomassie blue (Fig. 1a, e), comigrates with bovine serum albumin and probably represents a serum containment in those preparations. That protein is not present in the final preparations, however (Fig. 1c). In addition, the incorporation of both precursors indicated that the major bands contained both carbohydrate and protein. Colorimetric analysis of the protein mixtures by the Lowry test for protein⁶ and by a phenolsulphuric acid test for carbohydrate⁷ revealed a protein to carbohydrate mass ratio of about 10:1. These criteria suggested that the bands were composed of glycoproteins.

The similarities in the electrophoretic patterns suggested that released and extracted proteins were the same proteins and that the released proteins were components of the cell surface. That conclusion was supported by labelling whole cells with radioiodine using a lactoperoxidase system⁸ to ensure that only external components were radioactive. The electrophoretic pattern of proteins extracted from iodinated cells is shown in Fig. 1m. A duplicate culture of iodinated cells was washed to remove loosely attached peripheral components⁹ and incubated for 24 h. The iodinated released proteins (Fig. 1l) showed the same major bands as observed for extracted proteins. There were extra minor bands in the released protein fraction. The origin of those components is unknown.

Both extracted proteins and released proteins promoted the attachment and spreading of non-transformed cells. Table 1 shows the results of experiments in which low density cells were plated on glass in the absence of serum.

In these conditions very few cells attached and no cells had extended processes. The addition of bovine serum albumin 50 µg ml⁻¹ had no effect. However, the addition of 50 µg ml⁻¹ of extracted or released proteins resulted in > 90% of the cells spread within 20 h. The partial fractionation of the released and extracted proteins by ammonium sulphate precipitation showed that the activity was present in material that precipitates in a solution that is less than 35% saturated and that most of the activity is in the 20–35% saturated fraction (Table 1). Although this fractionation procedure is imperfect, it is reasonable to conclude that the spreading and attachment activities were associated with the presence of high molecular weight species. We were unable to detect any low molecular weight

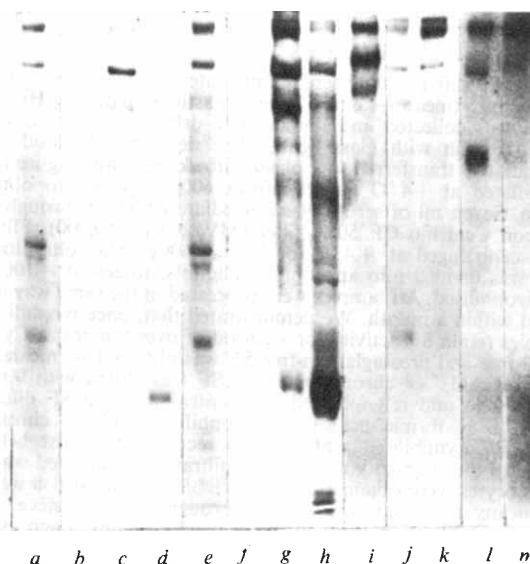


Fig. 1 Comparison of released and extracted proteins. All experiments used a single primary culture of human diploid fibroblasts that was derived from a foreskin biopsy and initiated in this laboratory. The cells were cultured in medium 199 (GIBCO) supplemented with 20% foetal bovine serum (Flow) and antibiotics. Released proteins were obtained as described previously¹. Extracted proteins were obtained by the method developed for chick cell surface proteins¹⁵ using 1 M urea. To obtain ¹⁴C-labelled proteins, confluent monolayers were pulsed for 24 h with 0.3–0.5 µCi ml⁻¹ ¹⁴C-amino acids (280 mCi mmol⁻¹) or ¹⁴C-glucosamine (60 mCi mmol⁻¹) (New England Nuclear). The monolayers were then washed to remove all traces of serum and the medium was replaced with serum-free medium 199 containing 0.3–0.5 µCi ml⁻¹ of the same labelled compound. After 24 h the released proteins were gently decanted and the cells were washed. Proteins were then extracted from the washed cells. ¹²⁵I-labelled released proteins were obtained by labelling a washed monolayer for 2 min with 500 µCi ml⁻¹ sodium iodide (NEN) using a lactoperoxidase-catalysed procedure⁸. The labelled cells were then incubated for 7–20 h, in serum-free medium 199. Electrophoretic separation of dithiothreitol-reduced samples was obtained by standard procedures¹⁶. Samples were heat denatured in 0.1% sodium dodecyl sulphate (SDS) and electrophoresed through a 5% polyacrylamide stacking gel (not shown) and a 7.5% polyacrylamide separating gel. This figure is a composite of several experiments performed at different times. Autoradiograms of the gels (tracks g–m) were obtained by exposing prefogged Kodak X-ray film to dried gels for 3–21 d at –60 °C. Protein concentrations in the 13 tracks range from 40 to 250 µg. Tracks a–e: Coomassie blue stained: a, released protein; b, 20% (NH₄)₂SO₄ released protein; c, 35% (NH₄)₂SO₄ released protein; d, 70% (NH₄)₂SO₄ released protein, and e, extracted protein. Tracks f–i: ¹⁴C-amino acid labelled: f, 20% (NH₄)₂SO₄ released protein; g, released protein; h, 70% (NH₄)₂SO₄ released protein, and i, 35% (NH₄)₂SO₄ extracted protein. Tracks j, k: ¹⁴C-glucosamine labelled: j, released protein and k, extracted protein. Tracks l and m: ¹²⁵I-labelled: l, released protein and m, extracted proteins. The three major bands near the top of the gels (shown most clearly in c) have apparent molecular weights > 150,000. The abbreviation AMS indicates that the protein was insoluble in the stated concentration of ammonium sulphate.

Table 1 Spreading activity of released and extracted proteins

Sample	No. of cells		% Spread
	Attached	Spread	
Control	7	0	0
+BSA	6	0	0
Released proteins	2	98	98
20% (NH ₄) ₂ SO ₄	28	27	49
35% (NH ₄) ₂ SO ₄	34	102	75
70% (NH ₄) ₂ SO ₄	9	1	10
Extracted proteins	4	98	96
35% (NH ₄) ₂ SO ₄	7	96	93

All numbers represent the means of at least two independent determinations. The control is medium 199 without protein. All other samples contain 35–45 µg of protein in medium 199. Released and extracted proteins were precipitated sequentially with 20%, 35%, and 70% saturated ammonium sulphate solutions at 0 °C. Attached and spread cells are determined by phase contrast microscopy according to the parameters suggested by Taylor¹⁷.

species on either 7.5% or 20% polyacrylamide gels (data not shown) even at concentrations as high as 5 mg ml⁻¹ (50 µg ml⁻¹ is required for > 95% spreading). Electrophoretic profiles of ammonium sulphate-precipitated materials (Fig. 1 *b–d*, *f–h*) indicated that the 20–35% fraction contained the three high molecular weight species.

The data may be interpreted as indicating either that the spreading activity and the attachment activity were due to separate components or that one protein promotes both activities at high concentration and only attachment at low concentrations. Table 2 shows that over 50% of the spreading activity was absorbed to fixed cells, but that cell attachment was unaffected by absorption. Trypsin-treated cells were washed and fixed before absorption to prevent the production and release of the proteins under investigation. The spreading activity was substantially reduced in the absorbed solution, but the total number of attached plus spread cells remained about the same for absorbed material as it was for non-absorbed material. Other experiments using radioactive released proteins showed that in the conditions of these experiments the proteins absorbed to the cells and not to the plastic container (data not shown). The data presented here show that cell surface components are active in cell attachment and spreading and that those components are released, in an active form, into the culture media. The function of that turnover is not known, however. We propose that the release and absorption of cell-surface components is a form of intercellular communication that promotes cell growth. Previously, we demonstrated

that cells plated at high density attached and spread in the absence of serum while low density cells required non-dialysable components present in conditioned medium¹. We suggest that at high density the concentration of cell surface-derived released proteins is high enough to maintain a critical concentration of those proteins on the cell surface. However, at low density, the equilibrium between the concentration of released proteins on the cell surface and in the culture medium is shifted so that the surface concentration is too low to support attachment and spreading. It is possible that the low-density cells have an altered physiology¹⁰, but our experiments do not test this.

We do not know which of the released proteins are responsible for the activities tested. However, the involvement of cell-surface components in cell adhesion is well established. A LETS/CSP protein has been shown to be active in red cell agglutination¹¹ and in the adhesion of transformed cells^{4,5}. In addition, there is considerable evidence to indicate that cell-membrane components are released into the culture media^{12,13} and that a serum component is antigenically identical to LETS/CSP¹⁴. It is possible that the large band at the top of the gel is identical to LETS/CSP³ and is responsible for some of the activity. However, it is also possible that more than one protein is involved since attachment and spreading activities seem to be due to separate components (Table 2).

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ALBERT J. T. MILLIS
MARIAN HOYLE

Department of Biological Sciences,
The University at Albany,
State University of New York,
Albany, New York 12222

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1. Millis, A. J. T., Hoyle, M. & Field, B. J. *Cell Physiol.* **93**, 17–24 (1977).
2. Moore, E. J. *Cell Biol.* **70**, 634–647 (1976).
3. Hynes, R. *Biochem. biophys. Acta* **458**, 73–107 (1976).
4. Yamada, K. M., Yamada, S. S. & Pastan, I. *Proc. natn. Acad. Sci. U.S.A.* **73**, 1217–1221 (1976).
5. Unnisa, J., Mautner, V., Lanza, R. & Hynes, R. *Cell* **11**, 115–126 (1977).
6. Lowry, O., Rosebrough, N. J., Farr, A. L. & Randall, R. J. *J. biol. Chem.* **193**, 265–275 (1951).
7. Ashwell, G. *Meth. Enzym.* **8**, 85–95 (1966).
8. Kennel, S. J., DelVillano, B. C. & Lerner, R. A. *Meth. molec. biol.* **6**, 1–38 (1973).
9. Singer, S. J. & Nicolson, G. *Science* **175**, 720–721 (1972).
10. Eagle, H. & Piez, K. J. *J. exp. Med.* **116**, 29–43 (1962).
11. Yamada, K. M., Yamada, S. S. & Pastan, I. *Proc. natn. Acad. Sci. U.S.A.* **72**, 3158–3162 (1975).
12. Doljanski, F. & Kapeller, M. J. *theor. Biol.* **62**, 253–270 (1976).
13. Schubert, D. *Expl Cell Res.* **102**, 329–340 (1976).
14. Rouslahti, E. & Vaheri, A. *Nature* **248**, 789–791 (1974).
15. Yamada, K. M. & Weston, J. A. *Proc. natn. Acad. Sci. U.S.A.* **71**, 3492–3496 (1974).
16. Laemmli, U. *Nature* **227**, 680–685 (1970).
17. Taylor, A. C. *Expl Cell Res. Suppl.* **8**, 154–173 (1961).

Table 2 Absorption of released protein spreading activity

Sample	Attached + spread = total			% Spread
Control	8	0	8	0
Released protein (50 µg ml ⁻¹)	3	100	103	97
Absorbed solution	60	49	109	45
Released protein (25 µg ml ⁻¹)	31	58	89	65
Absorbed solution	80	27	107	25

Values represent the means of two determinations per sample. Absorption procedure: released proteins were absorbed to whole fixed cells by incubating a cell suspension (1.8×10^7 cells per ml of 50 µg released protein) for 10 min at 37 °C. After the incubation the mixture was centrifuged to pellet the cells and the supernatant fraction retained as the 'absorbed solution'. The cells used for absorption had been treated with 50 µg ml⁻¹ trypsin solution for 10 min at 37 °C and subsequently fixed in 3% formaldehyde. Fixed cells were washed extensively and used without further delay. Residual proteolytic activity was not detected.

Calcium effects on gap junction structure and cell coupling

PROTOPLASMIC molecules of low molecular weight are known to travel freely across the boundaries between most neighbouring cells through small intercellular channels generally thought to be at the intramembranous particles of gap junctions^{1–3}. The permeability of the channels can be decreased to a complete interruption of cell communication by a variety of treatments^{2–10}. Most of these treatments raise the concentration of ionised calcium, [Ca²⁺], in the cytoplasm⁸, suggesting that calcium may be the uncoupling agent in both vertebrate⁹ and invertebrate cells^{2,8}. In some systems glutaraldehyde fixation has also been shown to produce uncoupling³; however, this type of uncoupling does not seem to be triggered by calcium¹⁰. In parallel with functional uncoupling reversible structural changes have been described in gap junctions of crayfish characterised by an increase in tightness and regularity of particle aggregation

and a decrease in junctional thickness and particle size¹⁰. These changes, interpreted as reflecting conformational rearrangements in the protein framework of the channels resulting in channel obliteration, were recently confirmed in the rat¹¹ although in the latter, functional uncoupling was not measured electrophysiologically, but only presumed on the basis of reasonable comparative arguments. It remains uncertain whether or not the changes in both the gap junction structure and the cell coupling are due to a direct action of calcium on the junctional membranes. We show here that the junctional change is indeed a calcium effect, triggered by $[Ca^{2+}]$ as low as 5×10^{-7} M.

Gap junctions of the eye lens of calf were studied by freeze-fracture either in intact cells or in isolated preparations. The lenses were dissected from the eyes 30–60 min after death. For experiments on intact cells the lenses were fixed in glutaraldehyde, infiltrated with glycerol (as cryoprotective agent) and processed for freeze-fracture as described elsewhere¹¹. In these preparations, gap junctions between epithelial cells of the anterior lens surface showed particles and complementary pits organised in regular hexagonal arrays with unit cells of ~ 8.5 nm (Fig. 1a), the crystallinity of the array being more pronounced in the pitted face, as described in other gap junctions¹², while gap junctions between fibre cells displayed particles and pits irregularly packed at various spacings (Fig. 1b). This difference, reported also by Benedetti *et al.*¹³ is probably a consequence of the difference in metabolic properties between epithelial and fibre cells. The tightly and regularly packed epithelial junctions are identical to rat liver and stomach junctions uncoupled by inhibitors of the metabolism and are likely to be in an uncoupled state as well, since the lens epithelium, known to possess an active oxidative metabolism¹⁴, is expected to suffer the effects of severe hypoxia if it is not fixed immediately after animal death. In fact the inhibition of the metabolism and the consequential depletion of energy stores caused by hypoxia¹⁵ are known to raise the cytoplasmic $[Ca^{2+}]$ and result in cell uncoupling⁸ as Ca^{2+} diffuses in the cytosol from the mitochondria and the surface membrane¹⁶. Fibre cells, on the other hand, derive their energy requirement from glycolytic metabolism¹⁴ and therefore are rather insensitive to hypoxia; this is probably the reason that their gap junctions display structural characteristics of normally coupled junctions.

For studies on isolated fibre cell junctions, eight to ten lenses per experiment were homogenised either in sodium bicarbonate buffer ($1 \cdot 10^{-3}$ M, $pH = 7.4$) or in the same buffer containing EDTA 1×10^{-2} M to chelate divalent cations. The homogenate was washed four times in bicarbonate or bicarbonate-EDTA buffer respectively, centrifuged to a pellet and freeze-fractured after glutaraldehyde fixation and cryoprotective treatment. Due to the great number of gap junctions between fibre cells, the

Fig. 1 Freeze-fracture replicas of gap junctions in intact cells of calf lens. *a*, Gap junction between epithelial cells of the anterior lens surface. Particles and complementary pits form a regular hexagonal array with a unit cell of ~ 8.5 nm. *b*, Gap junction between fibre cells. Particles and pits are randomly distributed at variable spacings. P, face P (protoplasmic membrane leaflet); E, face E (exoplasmic membrane leaflet). ($\times 160,000$).

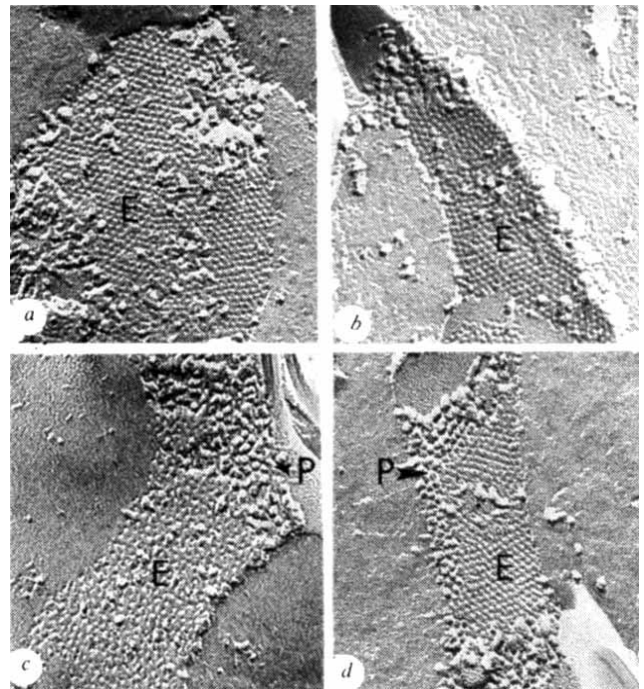
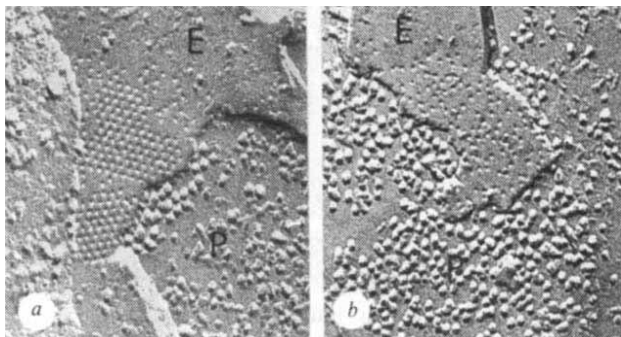


Fig. 2 Freeze-fracture replicas of gap junctions isolated from fibre cells of calf lens. *a*, Junction isolated in absence of EDTA. *b*, Junction isolated in absence of EDTA and subsequently incubated in 3×10^{-3} M EGTA. *c*, Junction isolated in presence of 1×10^{-2} M EDTA. *d*, Junction isolated in presence of 1×10^{-2} M EDTA and subsequently incubated in a 1×10^{-3} M calcium solution. Notice that junction isolation in absence of chelators of divalent cations causes aggregation of junctional particles into tight hexagonal arrays with unit cells of 7–8 nm (*a*). The phenomenon is not reversed by EGTA (*b*) or EDTA treatment, but can be prevented if the junctions are isolated in presence of EDTA (*c*). Exposure of junctions isolated in EDTA to calcium solutions causes hexagonal packing of the junctional particles (*d*). P, face P; E, face E. ($\times 160,000$).

crude pellets obtained are sufficiently rich in junctions not to require detergent treatment, and thus offer an excellent source of relatively undamaged gap junctions. Junctions isolated without EDTA display particles and pits tightly and regularly packed into hexagonal arrays with unit cells of 7–8 nm (Fig. 2a) while junctions isolated in bicarbonate-EDTA buffers have particles and pits loosely and irregularly aggregated (Fig. 2c). If junctions isolated in the presence of EDTA are suspended for 30 min at $37^\circ C$ in calcium solutions (1×10^{-3} – 3×10^{-3} M), buffered to $pH 7.4$ with 1×10^{-3} M Tris, particles and pits aggregate tightly into regular hexagonal arrays (Fig. 2d). Control junctions, suspended for the same length of time and at the same temperature in 1×10^{-2} M EDTA solutions ($pH = 7.4$) maintain the irregularly and loosely packed particle arrays (same as Fig. 2c). Junctions treated with calcium solutions do not recover the control appearance if they are subsequently exposed to 1×10^{-2} M solutions of EDTA or 3×10^{-3} M EGTA (Fig. 2b).

In junctions with regular hexagonal arrays the particles are ~ 1 nm smaller than in the others and appear more homogeneous in size and shape. In addition, in these junctions the fracture plane tends to remain within the same membrane, while in the irregularly packed junctions it steps frequently up and down from one to the other membrane. In most junctions there are a few particles which fracture away with the external leaflet, appearing as bumps on face E (Figs 2, 3). Particles on the pitted face of gap junctions are uncommon in vertebrates but are frequent in crayfish¹⁷; in the rat they are a common feature of gap junctions of livers perfused with hypertonic sucrose¹¹.

The finding of hexagonal particle packings in fibre cell junctions isolated in the absence of EDTA (Fig. 2a) suggested that trace amounts of divalent cations may be sufficient to cause the particle-clumping phenomenon. Therefore experiments using

EDTA-calcium buffers were performed in an attempt to define the triggering $[Ca^{2+}]$. Junctions isolated in bicarbonate-EDTA solutions, as previously described, were incubated for 30–120 min at 37 °C in calcium-EDTA solutions in which $[Ca^{2+}]$ was buffered to different levels ranging from $1 \times 10^{-8} M$ to $1 \times 10^{-6} M$. Calcium-EDTA solutions were prepared by dissolving calculated amounts of $CaCl_2$ into $1 \times 10^{-2} M$ solutions of EDTA buffered to pH 7 with $2 \times 10^{-2} M$ HEPES (Sigma). The amounts of $CaCl_2$ to be added were calculated using an EDTA dissociation constant for calcium of $5 \times 10^{-8} M$ (at pH 7, $\log K = 7.3$) (ref. 18). The following equation was used:

$$[Ca^{2+}]_i = ([Ca^{2+}]_f[EDTA]_i + [Ca^{2+}]_f^2 + [Ca^{2+}]_f K_D) / (K_D + [Ca^{2+}]_f)$$

where $[Ca^{2+}]_i$ represents the amount of total calcium per unit volume of medium, which gives a concentration of free calcium, $[Ca^{2+}]_f$, when the total amount of EDTA per unit volume of medium is $[EDTA]_i$, and K_D is the dissociation constant of the reaction: $Ca + EDTA \rightleftharpoons Ca - EDTA$. In each experiment the junctions were washed, before incubation, in the same calcium-EDTA solution to be used for incubation, so that residual amounts of EDTA left in the same sample after the preliminary isolation procedure would not interfere with the EDTA-calcium buffers. To make certain that changes in pH did not take place during incubation, the pH of the solutions was measured both at the beginning and at the end of the incubation period. No effect was detected on the structure of the junctions with solutions in which the $[Ca^{2+}]$ was buffered to values ranging from $1 \times 10^{-8} M$ to $3 \times 10^{-7} M$ (Fig. 3a, b), while solutions of $5 \times 10^{-7} M$ (Fig. 3c) or greater (Fig. 3d) $[Ca^{2+}]$ consistently caused the junctional particles and pits to aggregate tightly into regular hexagonal arrays. 89.3% Of all the junctions exposed to $[Ca^{2+}]$ greater than $5 \times 10^{-7} M$ showed hexagonal particle packing (13 experiments were performed and 131 junctions were photographed), and 99% of all the junctions exposed to $[Ca^{2+}]$ lower than $5 \times 10^{-7} M$ showed

irregular particle packing (11 experiments were performed and 114 junctions were photographed). Interestingly, the threshold of intracellular $[Ca^{2+}]$ for functional uncoupling is believed to be somewhere between $1 \times 10^{-7} M$ and $5 \times 10^{-6} M$ in salivary gland cells of insect larvae^{19,20} and slightly larger than $2 \times 10^{-7} M$ in cow and calf heart muscle²¹ (because of the close relationship between the onset of uncoupling and the onset of muscle tension) which fits reasonably well with $5 \times 10^{-7} M$ $[Ca^{2+}]$ obtained for the changes in gap junction structure.

In conclusion, the exposure of isolated gap junctions to $5 \times 10^{-7} M$ or greater $[Ca^{2+}]$ affects the junctional structure similarly to uncoupling treatments performed on intact cells. Since uncoupling is believed to be triggered by an increase in $[Ca^{2+}]$ in the cytoplasm, the calcium dependent changes in gap junction structure described here confirm the hypothesis^{10,11} that gap junctions with tightly packed hexagonal arrays of particles correspond to non-permeable junctions and indicate that calcium affects both the structure of the junctions and the cell coupling by acting directly on the junctional molecules. These data also suggest that small fluctuations from the normal intracellular $[Ca^{2+}]$, believed to be $1 \times 10^{-7} M$ (ref. 16), could be sufficient to produce functional uncoupling. Our findings do not, however, necessarily indicate that calcium is indeed the only agent capable of triggering the junctional changes with uncoupling. A recent report²² suggesting the uncoupling effects of low pH poses the question as to whether or not the structure of the junctions can also be affected in a similar way by acidic free calcium solutions. Experiments using such solutions are underway. Evidence from ultrastructure^{10,11} and X-ray diffraction^{23,24} suggests that the structural change is not a mere modification in particle packing but rather a conformational rearrangement in the molecular framework of the channels. The mechanism that causes the rearrangements in particle array is not known; however, it is interesting that it is triggered by a $[Ca^{2+}]$ similar to that²⁵ which causes muscle contraction. Although the presence of an actomyosin-like system associated with gap junction membranes has never been suggested, there are no reasons for discarding it *a priori*.

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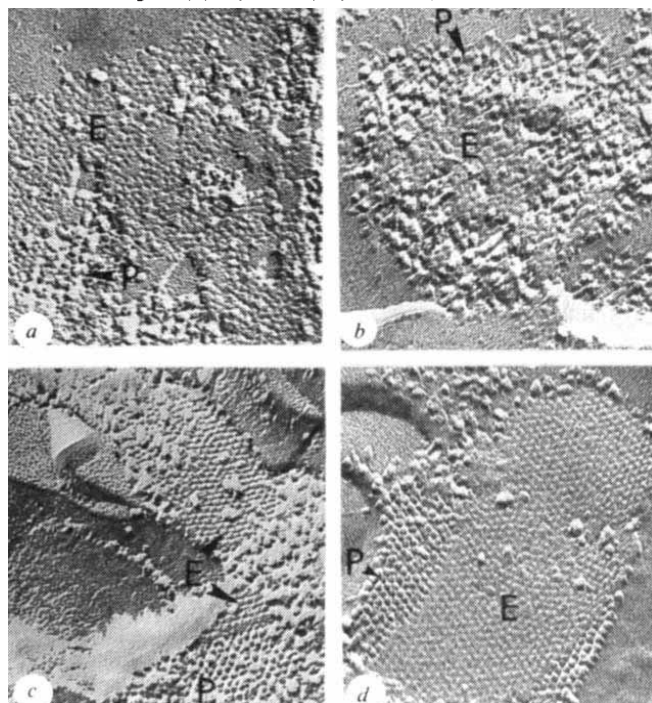
CAMILLO PERACCHIA

Department of Physiology,
University of Rochester,
School of Medicine and Dentistry,
601 Elmwood Avenue,
Rochester, New York 14642

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1. Peracchia, C. *Trends Biochem. Sci.* **2**, 26–30 (1977).
2. Loewenstein, W. R. *Cold Spring Harb. Symp. quant. Biol.* **40**, 49–63 (1975).
3. Bennett, M. V. L. *Fed Proc.* **32**, 65–75 (1973).
4. Barr, L., Dewey, M. M. & Berger, W. J. *gen. Physiol.* **48**, 797–823 (1965).
5. Dreifuss, J. J., Girardier, L. & Forssmann, W. G. *Pfluegers Arch. Ges. Physiol.* **292**, 13–33, (1966).
6. Loewenstein, W. R., Nakas, M. & Socolar, S. J. *J. gen. Physiol.* **50**, 1865–1891 (1967).
7. Asada, Y., & Bennett, M. V. L. *J. Cell Biol.* **49**, 159–172 (1971).
8. Rose, B. & Loewenstein, W. R. *Nature* **254**, 250–252 (1975).
9. DeMello, W. C. *J. Physiol., Lond.* **250**, 231–245 (1975).
10. Peracchia, C. & Dulhunty, A. F. *J. Cell Biol.* **70**, 419–439 (1976).
11. Peracchia, C. *J. Cell Biol.* **72**, 628–641 (1977).
12. Chalcraft, J. P. & Bullivant, S. J. *Cell Biol.* **47**, 49–60 (1970).
13. Benedetti, E. L. *et al. Biochim. biophys. Acta* **457**, 353–384 (1976).
14. Papaconstantinou, J. *Science* **156**, 338–346 (1967).
15. Lowry, O. H., Passonneau, J. U., Hasselberger, F. H. & Schulz, D. W. *J. biol. Chem.* **239**, 18–30 (1964).
16. Blaustein, M. P. *Rev. Physiol. Biochem. Pharmac.* **70**, 33–82 (1974).
17. Peracchia, C. *J. Cell Biol.* **57**, 66–76 (1973).
18. Chaberek, S. & Martell, A. E. *Organic Sequestering Agents* (Wiley, New York, 1959).
19. Déléze, J. & Loewenstein, W. R. *J. Membrane Biol.* **28**, 71–86 (1976).
20. Rose, B., Simpson, I. & Loewenstein, W. R. *Nature* **267**, 625–627 (1977).
21. Weingart, R. *J. Physiol., Lond.* **264**, 341–365 (1977).
22. Turin, L. & Warner, A. *Nature* **270**, 56–57 (1976).
23. Caspar, D. L. D., Goodenough, D. A., Makowski, L. & Phillips, W. C. *J. Cell Biol.* **74**, 605–628 (1977).
24. Makowski, L., Caspar, D. L. D., Phillips, W. C. & Goodenough, D. A. *J. Cell Biol.* **74**, 629–645 (1977).
25. Ashley, C. C., Caldwell, P. C., Lowe, A. G., Richards, C. D. & Schirmer, H. *J. Physiol., Lond.* **179**, 32P–33P (1965).

Fig. 3 Freeze-fracture replicas of gap junctions isolated from fibre cells of calf lens. All the junctions have been isolated in presence of $1 \times 10^{-2} M$ EDTA and subsequently washed and incubated in calcium-EDTA solutions (pH 7.0) in which $[Ca^{2+}]$ was buffered to $1 \times 10^{-7} M$ (a), $3 \times 10^{-7} M$ (b), $5 \times 10^{-7} M$ (c) and $1 \times 10^{-6} M$ (d). Note that particles and pits aggregate tightly into regularly hexagonal arrays only at a $[Ca^{2+}]$ of $5 \times 10^{-7} M$ (c) or higher (d). P, face P; E, face E. ($\times 160,000$).

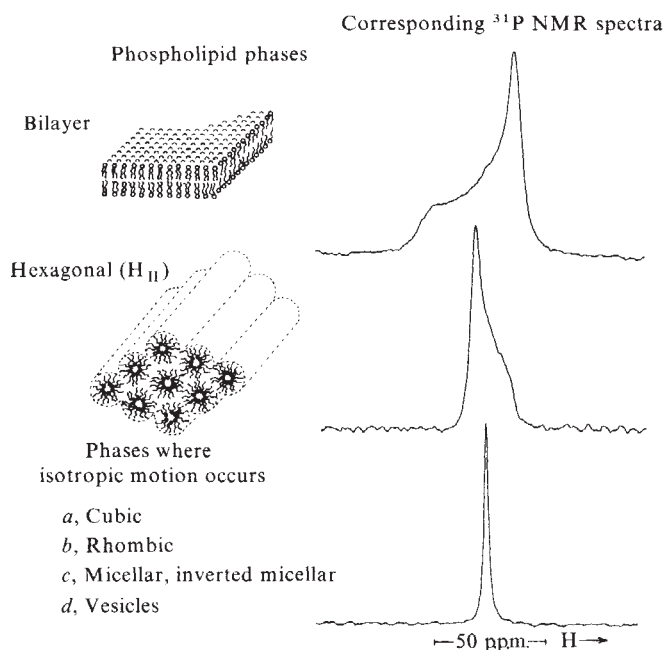


Effects of fusogenic agent on membrane structure of erythrocyte ghosts and the mechanism of membrane fusion

MEMBRANE fusion clearly requires that participating lipids assume some transitory non-bilayer configuration during the intermediate stages. Previous workers have suggested that intermediate micellar¹ or inverted micellar^{2,3} structures may occur, but the precise nature of possible intermediates and their relation to the physical properties of membrane lipids are obscure. In this regard, Lucy and co-workers⁴⁻⁸ have shown that 'fusogenic' agents such as fatty acids and their derivatives induce erythrocytes to fuse. Such agents might possibly promote fusion by enabling endogenous lipids to assume non-bilayer configurations. We have therefore investigated the influence of two such fusogens on the structure of the erythrocyte (ghost) membrane using ³¹P NMR techniques, which have been found to be sensitive to phospholipids in non-bilayer phases^{9,10}. We show that the incorporation of oleic acid and glycerol mono-oleate into the ghost membrane, at concentrations similar to those needed to induce cell fusion between erythrocytes *in vitro*, produce a well-defined transition of a variable portion of the membrane phospholipids from the bilayer phase to an hexagonal (H_{II}) phase. These results lead us to propose a model for membrane fusion induced by oleic acid, which we suggest may also apply to fusion events *in vivo*.

The phases available to hydrated liquid crystalline phospholipids, and the corresponding ³¹P NMR spectra obtained are illustrated in Fig. 1. Briefly, the phospholipids in (large) bilayer structures give rise to broad asymmetric ³¹P NMR spectra with a low field shoulder⁹⁻¹⁴. Alternatively, phospholipids in the hexagonal (H_{II}) phase (ref. 15) exhibit narrower

Fig. 1 Phospholipid phases and corresponding (36.4 MHz) ³¹P NMR spectra observed. The 36.4 MHz ³¹P NMR spectra were obtained from (unsonicated) aqueous dispersions of egg yolk lecithin at 30 °C (bilayer phase), soya phosphatidylethanolamine at 30 °C (hexagonal H_{II} phase) and a mixture of soya phosphatidylethanolamine and 15 mol% egg yolk lecithin at 30 °C (cubic, rhombic or inverted micellar phase, for fuller details see ref. 10). All aqueous dispersions contained 25 mM Tris-HAc (pH 7.1) and 2 mM EDTA. All spectra were obtained in the presence of high power (18 W) broad band proton decoupling.



spectra with a high field shoulder¹⁰. Finally, phospholipids in other available phases exhibit much narrower symmetrical ³¹P NMR spectra¹⁰.

The 36.4 MHz ³¹P NMR spectra obtained from erythrocyte ghosts incubated in the presence of increasing amounts of oleic acid are shown in Fig. 2. A progressive conversion of the membrane phospholipids from the bilayer phase to the hexagonal (H_{II}) phase is observed. Similar effects were also observed for glycerol mono-oleate. Such spectra cannot arise from lipids in

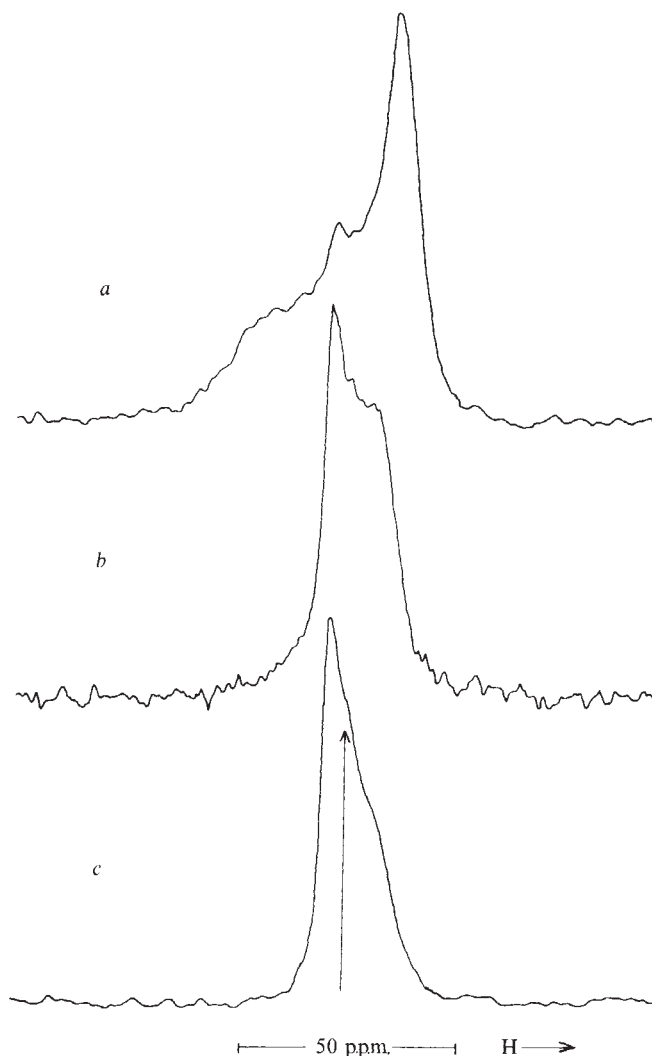


Fig. 2 36.4 MHz ³¹P NMR spectra obtained at 37 °C from a, 150 mg (dry weight) erythrocyte ghost incubated in medium A (for details of incubation conditions and composition of medium A, see below) in the absence of oleic acid; b, 150 mg (dry weight) erythrocyte ghost incubated in medium A containing 35 mg oleic acid; c, 150 mg (dry weight) erythrocyte ghost incubated in medium A containing 50 mg oleic acid. The arrow indicates the position of ³¹P NMR spectra arising from sonicated vesicles. Erythrocyte ghosts were prepared from human bank blood as detailed previously²⁴ and were subsequently lyophilised and stored under N₂ at -20 °C before use. Medium A contained NaCl (0.12 M), KCl (6 mM), MgSO₄·7H₂O (5 mM), CaCl₂·H₂O (2 mM) and Tricine (20 mM), and the pH adjusted to 6.8. The oleic acid was added drop by drop to 40 ml of medium A and subsequently sonicated (5 min) to obtain a milky suspension which was added to the (dry) ghost material. The resulting mixture was incubated at 37 °C for 20 min while stirring continuously. The material was subsequently centrifuged (50,000g) for 10 min at 0 °C and 0.1 ml D₂O (25 mM Tris-HAc, pH 7.0) added to the pellet, which was then transferred to the NMR tube. Spectra were obtained from up to 40,000 transients in the presence of high power (20 W) proton decoupling, using a 45° resonance frequency pulse and a 0.18 s interpulse time. Fatty acid determinations (see text) indicated an oleic acid to phospholipid ratio (mol/mol) of 1.3 for sample (b) and 2.4 for sample (c).

micellar or inverted micellar phases, or from bilayer phase lipids in rapid exchange with such micellar phases, as either situation would give rise to a narrow spectral component (≈ 20 Hz linewidth) with a chemical shift characteristic of isotropic averaging. Freeze fracture electron micrographs obtained from the sample of Fig. 2c reveal (Fig. 3) that the protein is segregated away from regions of the H_{II} phase, which exhibit a characteristic striated pattern consistent with previous freeze fracture visualisations of the H_{II} phase in model systems¹⁶.

The formation of the H_{II} phase was observed to be strongly dependent on the presence of Ca^{2+} in the incubation medium. Incubations equivalent to that performed for the sample of Fig. 2c but where Ca^{2+} was omitted resulted in broad, rather featureless ^{31}P NMR spectra which could not be clearly identified with either the bilayer or H_{II} phases. Control experiments (see below) showed that similar concentrations of oleic acid were associated with the ghost membrane in both situations. ^{31}P NMR studies were also performed on liposomes composed of the total lipids extracted from the erythrocyte membrane (to which various amounts of fusogen had been added prior to hydration) in the presence and absence of Ca^{2+} . Addition of Ca^{2+} to these systems promoted the formation of the H_{II} phase (above a fusogen/phospholipid ratio of 1 mol/mol). This provides strong evidence that Ca^{2+} directly facilitates the formation of the H_{II} phase in the ghost membrane. This apparent ability of Ca^{2+} to induce bilayer to hexagonal (H_{II}) phase transitions may be related to the previously noted^{17,18} ability of Ca^{2+} to cause lateral phase separation of charged lipid species in mixed lipid systems.

If the H_{II} phase formed in the fusogen-treated erythrocyte ghost membranes is relevant to the mechanism by which fusogens facilitate fusion between erythrocytes *in vitro*, similar amounts of fusogen should be associated with ghosts and erythrocytes when significant hexagonal (H_{II}) phase or fusion, respectively, is observed. Thus fusion was induced between erythrocytes, employing oleic acid, in the manner detailed previously⁷. The cells were centrifuged on observation of initial fusion events (10–20% fusion, as observed in a phase contrast microscope), and a fatty acid analysis of the pellet allowed a determination of the oleic acid associated with the cells. Excess fatty acid floated on the supernatant after centrifugation. Approximately 1 mol of oleic acid per mol of erythrocyte phospholipid was required before significant fusion occurred. This may be compared to an oleic acid/phospholipid ratio of 1.3 (mol/mol) obtained for the sample of Fig. 2b where significant formation of the H_{II} phase is observed. Assuming that a similar proportion of the membrane associated fusogen is contained in the ghost and erythrocyte bilayers respectively, these results indicate that membrane concentrations of fusogen sufficient to initiate fusion in erythrocytes are also sufficient to promote formation of the H_{II} phase in the ghost membrane. This suggests that observation of the H_{II} phase in the ghost system corresponds to ghost fusion (although this has not yet been observed by other techniques) and also suggests that formation, or a tendency to formation, of the H_{II} phase in the erythrocyte membrane itself is vital to subsequent fusion events.

On the basis of this information we propose a model of cell fusion induced by oleic acid which is illustrated in Fig. 4. The fusion event is envisaged to proceed through (at least) four stages; these involve aggregation of protein to produce areas of (protein free) lipid bilayer¹⁹, which subsequently make contact (Fig. 4b). It is then suggested that the two outer monolayers combine to form an intermediate hexagonal H_{II} phase (Fig. 4c) which is subsequently restabilised to bilayer structure (Fig. 4d) to complete the fusion process. The presence of Ca^{2+} is probably important at each stage, indeed Ca^{2+} has already been implicated in the processes of protein aggregation and cell-cell contact²⁰. We emphasise, however, that the presence of Ca^{2+} would seem to be particularly important for the formation of the H_{II} phase of Fig. 4c. Further, the resta-



Fig. 3 Freeze-etch electron micrograph obtained from erythrocyte ghost sample giving rise to the spectra of Fig. 2c. The sample was quenched from 20 °C. Other conditions were as detailed previously²⁵. Magnification $\times 250,000$.

bilisation event may be envisaged to occur by way of an active (ATP-dependent) removal of Ca^{2+} from the cytoplasm of the partially fused cells of Fig. 4c, thus producing a strong chemical potential between the aqueous pores of the H_{II} phase and the cytoplasm. This would encourage the removal of Ca^{2+} and subsequent reversion of the H_{II} lipid to the bilayer phase.

There are strong indications that the mechanism of membrane fusion shown in Fig. 4 may also apply to naturally occurring membrane fusion *in vivo*. In particular, many species of naturally occurring lipids found in significant concentrations in biological membranes prefer the hexagonal (H_{II}) phase or other inverted phases (intermediate formation of inverted micelles would be equally acceptable to the fusion model of Fig. 4) over bilayer structure. Notable examples include unsaturated phosphatidylethanolamines^{9,10,21,22} and cardiolipin²³. Moreover, the formation of H_{II} phases for both these lipid species may be facilitated by the presence of Ca^{2+} (ref. 23 and P. R. Cullis, unpublished). Cholesterol also tends to favour the formation of hexagonal (H_{II}) phases in certain situations¹⁰. Thus 'natural fusogens' abound, and segregation of such non-bilayer lipids into particular regions of the membrane (in the presence of Ca^{2+}) would be expected to facilitate fusion events. Cell-cell contact in the form of Fig. 4c may also correspond to 'quasi-fusion' events²⁰ associated with tight junctions.

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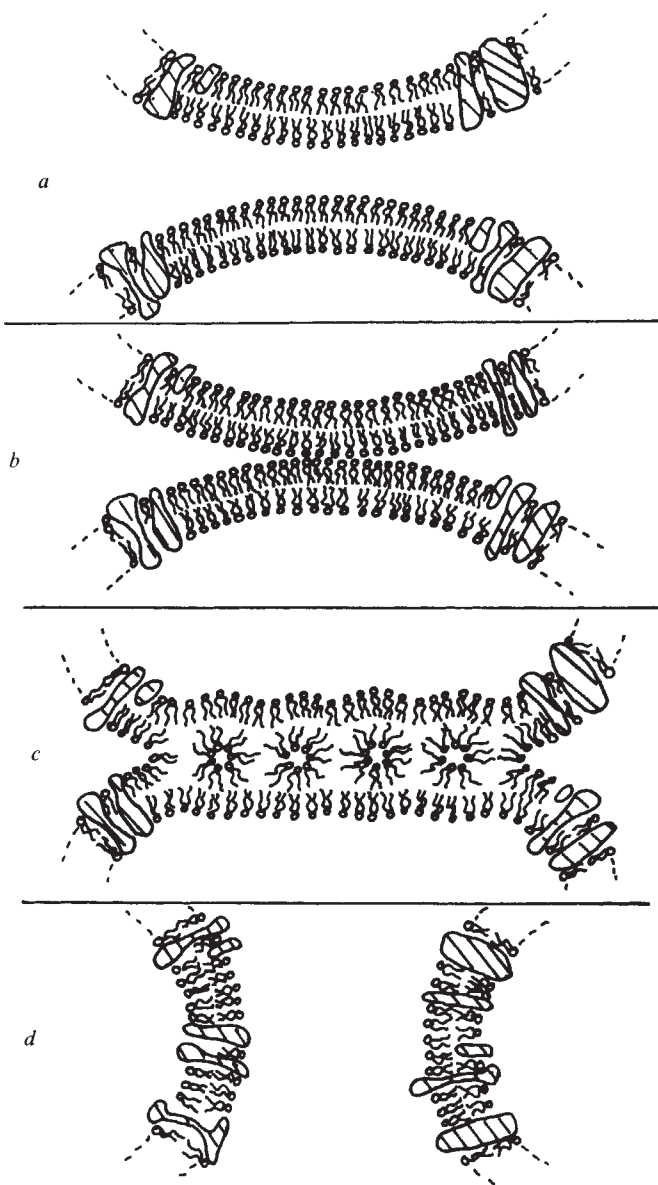


Fig. 4 Proposed mechanism of membrane fusion. The slashed areas traversing the membrane indicate integral membrane protein. Note that the diameter of the aqueous channels of the hexagonal phase component of part *c* are drawn approximately to scale with respect to the thickness of the bilayer.

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P. R. CULLIS

Biochemistry Department,
University of British Columbia,
Vancouver V6T 1W5, Canada

M. J. HOPE

Biochemisch Laboratorium,
Rijksuniversiteit te Utrecht,
Transitorium 3, Padualaan 8, 'De Uithof',
Utrecht, The Netherlands

Received 8 July; accepted 9 December 1977.

- Lucy, J. A. *Nature* 227, 814–817 (1970).
- Lau, A. L. Y. & Chan, S. I. *Proc. natn. Acad. Sci. U.S.A.* 72, 2170–2174 (1975).
- Pinto da Silva, P. & Nogueira, M. L. *J. Cell Biol.* 73, 161–181 (1977).
- Howell, J. I. & Lucy, J. A. *FEBS Lett.* 4, 147–150 (1969).
- Poole, A. R., Howell, J. I. & Lucy, J. A. *Nature* 227, 810–813 (1970).
- Ahkong, Q. F., Cramp, F. C., Fisher, D., Howell, J. I. & Lucy, J. A. *J. Cell Sci.* 10, 769–787 (1972).
- Ahkong, Q. F., Fisher, D., Tampion, W. & Lucy, J. A. *Biochem. J.* 136, 147–155 (1973).
- Lucy, J. A. in *The Structure of Biological Membranes* Nobel Symp. no. 34 (ed. Abrahamsson, S.) (Plenum, New York, 1976).
- Cullis, P. R. & de Kruijff, B. *Biochim. biophys. Acta* 436, 523–540 (1976).
- Cullis, P. R. & de Kruijff, B. *Biochim. biophys. Acta* (in the press) (1977).

- McLaughlin, A. C. *et al. FEBS Lett.* 57, 213–218 (1975).
- Cullis, P. R. & McLaughlin, A. C. *Trends biochem. Sci.* 2, 196–199 (1977).
- Gally, H. U., Niederberger, W. & Seelig, J. *Biochemistry* 14, 3647–3652 (1975).
- Kohler, S. J. & Klein, M. P. *Biochemistry* 16, 519–526 (1977).
- Luzzatti, V., Gulik-Krzywicki, T. & Tardieu, A. *Nature* 218, 1031–1034 (1968).
- van Dijk, P. W. M., de Kruijff, B., van Deenen, L. L. M., de Gier, J. & Demel, R. A. *Biochim. biophys. Acta* 455, 576–587 (1976).
- Ohnishi, S. I. & Ito, T. *Biochem. biophys. Res. Commun.* 51, 132–138 (1973).
- Papahadjopoulos, D., Poste, G., Schaeffer, B. E. & Vail, W. J. *Biochim. biophys. Acta* 352, 10–28 (1974).
- Ahkong, Q. F., Fisher, D., Tampion, W. & Lucy, J. A. *Nature* 243, 194–195 (1975).
- Poste, G. & Allison, A. C. *Biochim. biophys. Acta* 300, 421–465 (1973).
- Rand, R. P., Tinker, D. O. & Fast, P. G. *Chem. Phys. Lipids* 6, 333–342 (1971).
- Junger, E. & Reinauer, H. *Biochim. biophys. Acta* 183, 304–308 (1969).
- Rand, R. P. & Sengupta, S. *Biochim. biophys. Acta* 255, 484–492 (1972).
- Cullis, P. R. *FEBS Lett.* 68, 173–176 (1976).
- Ververgaert, P. H. J. Th., Verkleij, A. J., Elbers, P. F. & van Deenen, L. L. M. *Biochim. biophys. Acta* 311, 320–329 (1973).

Distribution of sialic acids on the red blood cell membrane in β thalassaemia

SIALIC acids (SA) of the red blood cell (RBC) membrane are considered to play an important part in the physiology of the RBC¹. There are indications that the amount of membrane sialic acid is a major factor in distinguishing young from aged RBC. Thus, ageing of circulating RBC is associated with a reduction of 20–30% SA which may be an important determinant of RBC survival^{2,3} in the eventual recognition and sequestration of the latter cells by the reticuloendothelial system². During studies of thalassaemic RBC we observed, among other alterations, that the average level of SA was approximately 25% less than in normal RBC membranes obtained from healthy donors⁴. We, therefore, investigated the comparative ultrastructural distribution of SA on the surface of thalassaemic RBC membranes. We found that the SA residues on the surface of thalassaemic RBC are distributed in an uneven manner and are less abundant than those present on normal RBC surfaces.

We used three cytochemical methods in conjunction with transmission electron microscopy, in order to detect SA on membranes of intact RBC. One method is based on the production of a biotinyl derivative of SA which can be visualised by ferritin-avidin conjugates as described previously^{5,6}. In addition, two other techniques were used, cationised ferritin (CF) (ref. 7) and ruthenium red⁸, both of which interact with negatively charged sites on the membrane. The latter techniques were used because membrane-based sialyl residues are known to contribute most of the negative charge on the surface of the human RBC⁹.

The results obtained from all three techniques are illustrated in Fig. 1. The distribution of the above membrane probes from representative thalassaemic RBC samples was compared with that of normal RBC. In the former cases, only cells with characteristic thalassaemic morphology^{10,11} were evaluated. Both normal and thalassaemic RBC stained with ruthenium red, and a continuous layer of electron-dense material was observed. The deposit was much less dense, however, on the surface of thalassaemic RBC (Fig. 1a) than on normal cell membranes (Fig. 1b).

Unlike the ruthenium red stain, which results in an amorphous layer of reaction products, ferritin-based stains are characterised by a particulate marker of uniform size. The ferritin label can be quantitatively evaluated by enumeration and enables a finer resolution of the given surface site. Thus, quantification of cationised ferritin (CF) on micrographs of tangentially sectioned membrane (Figs 1c and d) revealed less attached CF particles per μ^2 on thalassaemic cells ($1,200 \pm 275 \sigma$) than on normal RBC ($2,030 \pm 150 \sigma$). In some instances the CF label on the former cell membranes was interrupted by unlabelled gaps (Fig 1c, arrows), in contrasts to the more even distribution of the label on normal RBC.

Additional details of the distribution of membrane SA were obtained by a more specific technique using ferritin-

avidin conjugates to visualise biotinylated sialyl residues. Examination of perpendicularly sectioned membranes obtained by this technique reveals the topographical resolution of sialyl residues from the lipid bilayer^{6,7}. With the above method, ferritin particles on the surface membrane of thalassaemic RBC are distributed in patches separated by unlabelled gaps (Fig. 1e). In addition, a distance of up to 300 Å could be discerned between some of the aggregated ferritin particles and the plane of the membrane surface. These observations may indicate either that some SA residues on thalassaemic RBC are associated with oligosaccharides of a more complex nature or that these sites have been displaced from the membrane surface. These phenomena were absent in normal RBC labelled in the same manner. In the normal RBC membrane, ferritin particles were almost uniformly distributed and protruded about 50–100 Å from the outer electron-dense layer of the cell membrane (Fig. 1f).

These ultrastructural observations support biochemical data which indicate that less SA are present on the surface membrane of thalassaemic RBC⁴. Similar observations on RBC membranes obtained from patients with β -thalassaemia intermedia are summarised in Table 1. Although the latter form of thalassaemia is morphologically indistinguishable from β -thalassaemia major, the clinical course is less severe, and the patients do not require

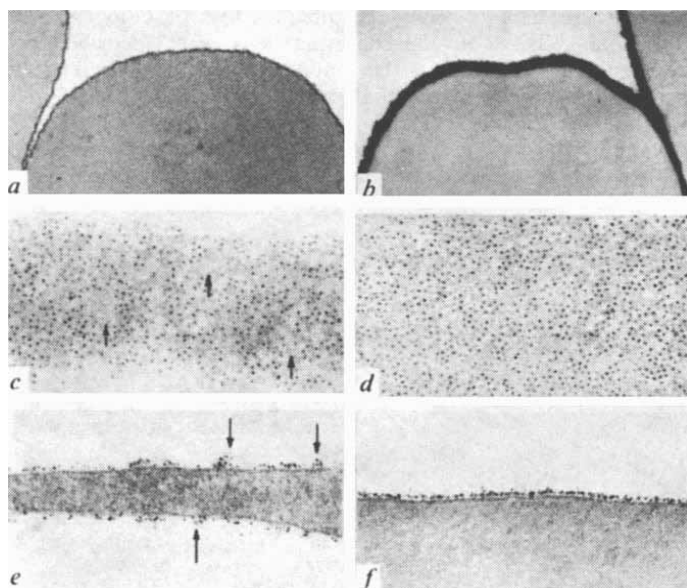


Fig. 1 Distribution of membrane probes on thin sections of thalassaemic (a, c, e) and normal (b, d, f) RBC. Thalassaemic RBC (a) stained with ruthenium red. Note the relatively thin layer of the reaction products when compared with normal RBC (b). Tangential section of thalassaemic RBC (c) treated with cationised ferritin (CF). The labelling pattern is interrupted by unlabelled areas (arrows). Quantification of the CF label on tangentially sectioned thalassaemic membranes showed reduction of about 40% compared with the uniformly CF-labelled normal RBC (d). Perpendicular section of thalassaemic RBC membrane (e) treated as follows: cells, washed in PBS, were reacted with 1 mM sodium periodate, washed twice, and treated with biotin hydrazide (2.5 mg ml⁻¹). Cells were then fixed for 30 min at room temperature with Karnovsky's fixative, labelled with ferritin-avidin conjugates (1 mg ml⁻¹ ferritin)^{6,7}, washed again, post-fixed with 1% osmium tetroxide and embedded in Epon 812. Perpendicular section of representative RBC membrane from healthy donors (f) treated in the same manner as in e. Note the presence of aggregates separated by unlabelled gaps in e, in addition to the distance (arrows) of some of the ferritin particles from the membrane surface. In contrast, a uniform labelling pattern is obtained on normal RBC membranes in f. Magnifications: a, b $\times 40,000$; c, d $\times 80,000$; e, f $\times 60,000$.

Table 1 Sialic acid content of thalassaemic and control RBC membranes

Membranes	($\mu\text{g mg}^{-1}$) Membrane protein
Control (9)	44.9 $\pm$ 2.5
β -Thalassaemia major (17)	28.4 $\pm$ 3.3
β -Thalassaemia intermedia (4)	27.3 $\pm$ 4.1

The RBC membranes were prepared as described by Kahane and Rachmilewitz⁴. Sialic acids were determined by the thiobarbituric acid procedure¹³. The number of samples tested is indicated in parenthesis.

frequent blood transfusions¹⁰. Nevertheless, the distribution of SA on the RBC surface in these patients was similar to that seen on β -thalassaemia major RBC membranes (Fig. 1e).

The underlying mechanism for the observed reduction in surface SA in thalassaemic RBC is still unknown. Several factors could be responsible for this phenomenon. Membrane biogenesis may be defective in precursor cells, leading to the release of RBC with altered content and distribution of SA. Alternatively, reduced SA levels could be attributed to an enhanced rate of their removal from thalassaemic RBC membranes. Thus, the short life-span of thalassaemic RBC¹⁰ may be related to an increased rate of sequestration due to the loss in surface SA.

Recently, Riggs and Ingram¹² have also reported lower levels of SA in RBC membranes of patients with sickle cell anaemia. In view of the results recorded here, it is possible that a reduction in SA may occur in other types of anaemia. The potential interrelationship between the reduced sialic acid content in the membrane and RBC pathology warrants further investigation.

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I. KAHANE

Biomembrane Research Laboratory,
The Hebrew University Hadassah Medical School

A. POLLIACK

Haematology Department,
Hadassah University Hospital

E. A. RACHMILEWITZ

Haematology Service,
Hadassah University Hospital,
Mount Scopus, Jerusalem

E. A. BAYER

Department of Biophysics,
The Weizmann Institute of Science

E. SKUTELSKY

Section of Biological Ultrastructure,
The Weizmann Institute of Science,
Rehovot, Israel

Received 3 September; accepted 7 December 1977.

- Weinstein, R. S. in *The Red Blood Cell* (ed. Surgenor, D. MacN.) 1, 213–268 (Academic, New York, 1974).
- Durocher, J. R., Payne, R. C. & Conrad, M. E. *Blood* **45**, 11–20 (1975).
- Jancik, J., Schauer, R. & Streicher, H. -J. *Hoppe-Seyler's Z. physiol. Chem.* **356**, 1329–1331 (1975).
- Kahane, I. & Rachmilewitz, E. A. *Israel J. med. Sci.* **12**, 11–15 (1976).
- Bayer, E. A., Skutelsky, E., Wynne, D. & Wilchek, M. *J. Histochem. Cytochem.* **24**, 933–939 (1976).
- Skutelsky, E., Danon, D., Wilchek, M. & Bayer, E. A. *J. ultrastruct. Res.* (in the press).
- Danon, D., Goldstein, L., Marikovsky, Y. & Skutelsky, E. *J. ultrastruct. Res.* **38**, 500–510 (1972).
- Luft, J. H. *Fedn Proc.* **25**, 1773–1783 (1966).
- Eylar, E. H., Madoff, M. A., Brody, O. V. & Oncley, J. L. *J. biol. Chem.* **237**, 1992–2000 (1962).
- Weatherall, D. J. & Clegg, J. B. *The Thalassaemia Syndromes* (Blackwell Scientific, London, 1972).
- Polliack, A. & Rachmilewitz, E. A. *Br. J. Haematol.* **24**, 319–326 (1973).
- Riggs, M. G. & Ingram, V. M. *Biochem. biophys. Res. Commun.* **74**, 191–198 (1977).
- Warren, L. *J. biol. Chem.* **234**, 1971–1975 (1951).

Intraventricular kainic acid preferentially destroys hippocampal pyramidal cells

THE hippocampus is particularly vulnerable to a variety of conditions, such as anoxia, status epilepticus and senile dementia, in which central neurones are lost^{1,2}. Most commonly, the lesion involves only the Sommer sector (h_1) and the endfolium (h_3 – h_5), sparing area h_2 , the fascia dentata and most regions outside the hippocampal formation. The consequences for hippocampal connections are unknown. Studies on the rat hippocampus suggest that connections made by the affected neurones could be replaced by axons of other neurones which project to the same areas^{3,4}. These anomalous synapses might either compensate in part for the loss of cells or contribute to whatever functional deficits may derive from the lesion. Since a good deal is known about afferent and efferent hippocampal connections in the rat, this animal might serve as a model for studies of hippocampal damage. However, the selective pathology seen clinically cannot be reproduced by conventional lesioning techniques. Ideally, one would like to use a toxin relatively specific for the neurones in question. Kainic acid, a potent excitatory analogue of glutamic acid^{5–7}, has been used to destroy neurones in the arcuate nucleus⁸ and striatum,^{9–11} while sparing fibres which pass to or through these regions. Previous workers have also briefly noted lesions in the hippocampus,^{8,11} but these were not described. Accordingly, we injected kainic acid intraventricularly into the rat brain and studied its effect on hippocampal neurones. We now report the unusual sensitivity of CA3–CA4, and to a lesser extent CA1, pyramidal cells to this agent. Our results suggest that kainic acid lesions can provide a model of hippocampal damage in man.

Male Sprague–Dawley rats (60–150-d-old) were anaesthetised with sodium pentobarbital and positioned in a stereotaxic apparatus (David Kopf Instruments) with the nose bar set at +5. A hole was drilled on bregma at 1.5 mm lateral to the midline, and a 1- μ l unbevelled Hamilton syringe was inserted to a depth of 4.7 mm from the surface of the skull. Kainic acid dissolved in 1 μ l of Elliott's artificial cerebrospinal fluid¹² (pH 7.3–7.5) was infused over a 30-min period. After 1–55 d the rats were killed by transcardial perfusion with neutralised formalin in normal saline, and the brains were examined histologically.

A modified Fink–Heimer method¹³ was used to detect degenerating neurones 1 and 3 d after kainic acid treatment. Argyrophilic (degenerating) hippocampal neurones (Fig. 1b) were seen at these survival times after unilateral injection of as little as 0.1 μ g (about 0.5 nmol). At this dose kainic acid killed only the pyramidal cells in area CA3a at the rostral pole of the hippocampus. Increasing doses destroyed pyramidal cells at increasingly caudal levels and in additional subfields. Cell destruction proceeded from the entrance of the fimbria (area CA3a) toward the dentate hilus (areas CA3c and CA4). At a dose of 0.8 μ g argyrophilic pyramidal cells were seen throughout areas CA3 and CA4 in all sections cut through the rostral half to two-thirds of the hippocampal formation (Fig. 1a). Intermediate patterns were obtained when 0.3 or 0.5 μ g was administered. Doses higher than 0.8 μ g were usually required to kill the pyramidal cells of area CA1, and the pyramids in area CA2 were seldom destroyed by any non-fatal dose (LD₅₀ of about 1.1 μ g). Dentate granule cells were destroyed only when 3 μ g of the drug was injected directly into the hippocampus. This hierarchy of sensitivity to kainic acid resembled that of hippocampal neurones to injurious conditions in man, and it could not be explained by the proximity of affected neurones to the lateral ventricle.

At 1 d after injection the degenerating elements were predominantly cell bodies and dendrites. By 3 d, however, we could identify a substantial number of degenerating axons and boutons. Kainic acid did not directly damage the septo-

hippocampal fibres (Fig. 2b) nor the hippocampal mossy fibres, whose cell bodies of origin remained intact. Thus, as in other regions,^{8,11,15} kainic acid selectively attacked cell bodies and dendrites, whereas axons of the same cells were only secondarily affected and afferent fibres were apparently unaffected.

In adjacent sections stained with cresyl violet degenerating cell bodies appeared severely shrunken, and their nuclei stained more darkly than normal (Fig. 1d). These neurones appeared to have been partially removed by 3 d after injection (Fig. 1c), although degenerating neurones could still be seen in some cases for at least 2 weeks. Over a period of several weeks areas of massive neuronal degeneration became gliotic and then atrophied (Fig. 2). Kainic acid exerted its toxic action directly on the hippocampus and not indirectly through an action elsewhere since the same histological results were obtained after intrahippocampal injection. That argyrophilic neurones actually degenerated and were not merely damaged reversibly was shown by the reduction or absence of neurones 1–2 months after injection in areas where argyrophilic cells were present at earlier times (Fig. 2a).

Argyrophilic neurones were not detected in regions other than hippocampus, when 0.1–0.3, and usually 0.5, μ g were administered, except for perhaps a few cells in the lateral septum immediately adjacent to the site of injection. Thus at these dosage levels kainic acid produced a fairly pure hippocampal lesion. With increasing dose, argyrophilic neurones were observed in additional regions, including some of the thalamic nuclei, amygdala and deep layers of the cerebral neocortex (but not striatum or hypothalamus). In man, cell loss in these regions often accompanies loss of hippocampal pyramidal cells.^{1,2} No degenerating cells were identified in areas known to project to the hippocampal formation, except in nucleus reuniens of the thalamus¹⁶ and in layer III of the entorhinal cortex.

Animals given intraventricular kainic acid showed symptoms of a partially suppressed convulsion lasting 3–4.5 h. At all doses

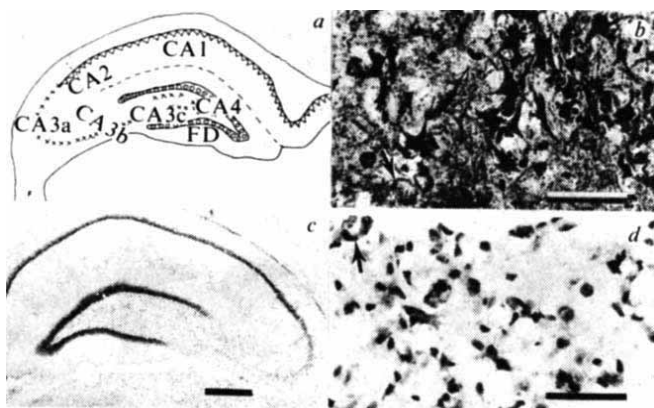


Fig. 1 Frontal sections through the dorsal rat hippocampal formation 3 d after ipsilateral intraventricular injection of 0.8 μ g of kainic acid. *a*, Diagram of hippocampal formation showing the location of degenerating pyramidal cells (X). The hippocampal formation is divided into fields according to Lorente de Nó²¹. Area CA1 corresponds roughly to h_1 in man, CA2 to h_2 , CA3 to h_3 and h_1 and CA4 to h_5 . Dashed lines denote the hippocampal fissure and the border of areas CA1 and CA2. FD, fascia dentata; F, fimbria; triangles, normal-appearing pyramidal cells; circles, dentate granule cells. A few scattered normal-appearing neurones were also found among the degenerating cells. *b*, High power view of part of area CA3c showing degenerating pyramidal cells. A few normal-appearing neurones (arrow) are also present. Fink–Heimer stain. Scale bar, 0.05 mm. *c*, Adjacent section stained with cresyl violet showing neuronal loss in areas CA3 and CA4. Note the glial proliferation in these areas. Scale bar, 0.5 mm. *d*, High power view of part of area CA3c showing darkly-stained shrunken pyramidal cells. Small dark bodies are probably glial cells. Some normal-appearing neurones (arrow) remain. Cresyl violet stain. Scale bar, 0.05 mm.

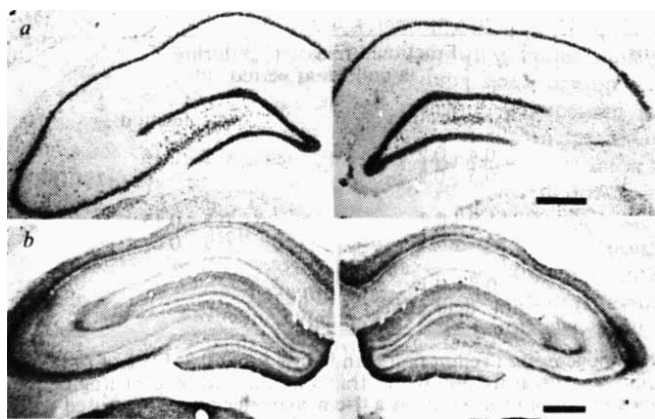


Fig. 2 Dorsal hippocampal formation 34 d after intraventricular injection of 0.8 μ g of kainic acid. Injected side on the right, contralateral side on the left. Ipsilateral areas CA3 and CA4 have atrophied noticeably by this time. *a*, Cresyl violet stain to show permanent loss of pyramidal cells in areas CA3 and CA4. Note persistent gliosis in affected areas. Scale bar, 0.5 mm. *b*, Acetylcholinesterase stain to show preservation of septohippocampal fibres^{25,26}. The density and distribution of these fibres is unaltered by kainic acid. Timm's sulfide silver staining of mossy fibres²⁷ showed those fibres were also unaffected. Scale bar, 0.5 mm.

these included vibrissa tremor and distended eye ipsilateral to the injection and at doses of 0.8 μ g and above there was in addition upper body tremor, rigidity of the limbs and foaming at the mouth. Several convulsants which interfere with GABA-mediated transmission and induce prolonged hippocampal seizures produce neuronal damage in baboons¹⁷⁻¹⁹ and cats²⁰ similar to that which kainic acid causes in rats. These agents probably induce a seizure through disinhibition, whereas kainic acid depolarises neurons directly. The similar pathology in these cases suggests that the cell loss in the present study resulted from a prolonged hippocampal seizure, similar to an episode of status epilepticus.

With regard to its mechanism of action, previous workers have assumed that kainic acid serves simply as a potent glutamate agonist, interacting with a glutamate receptor to effect a prolonged depolarisation. This chronic excitation would then lead to an irreversible ionic imbalance resulting in cell death. Indeed the destructive effects of the two amino acids on striatal and arcuate neurones appear very similar,^{8,10} although they are effective at doses about two orders of magnitude apart. In hippocampus, however, we have found glutamic acid to be virtually ineffective in destroying the pyramidal cells, even when it was injected directly into this region at a dose more than three orders of magnitude greater than the minimally effective dose of kainic acid. Furthermore, the dentate granule cells were quite resistant to the toxic effects of this agent, even though glutamate probably serves as a major excitatory transmitter in the fascia dentata²¹⁻²³ and the granule cells are readily depolarised by iontophoretically applied glutamate (unpublished observation). These results raise the possibility that either the glutamate receptors in the hippocampal formation differ from those in the striatum and arcuate nucleus or the destructive effects which kainic acid elicits in the hippocampus are not mediated through an interaction with a glutamate receptor.

Since kainic acid preferentially affects those neurones which are most susceptible to damage by a variety of adverse conditions, these lesions may provide a useful model of hippocampal injury in man. Kainic acid should prove advantageous in investigations of (a) the vulnerability of the hippocampus, particularly in status epilepticus, (b) the plasticity of hippocampal connections after brain damage and (c) the presently controversial behavioural role of the hippocampus.

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J. VICTOR NADLER
BRUCE W. PERRY
CARL W. COTMAN

Department of Psychobiology,
University of California,
Irvine, California 92717

Received October 3; accepted 21 November 1977.

1. Minckler, J. (ed.) *Pathology of the Nervous System* 2, (McGraw-Hill, New York, 1971).
2. Blackwood, W. & Corsellis, J. A. N. (eds) *Greenfield's Neuropathology* (Arnold, London, 1976).
3. Lynch, G. & Cotman, C. W. in *The Hippocampus* 1, (eds Isaacson, R. L. & Pribram, K. H.) 123-155 (Plenum, New York, 1975).
4. Cotman, C. W. & Lynch, G. S. in *Neuronal Recognition* (ed. Barondes, S. H.) 69-108 (Plenum, New York, 1976).
5. Shinozaki, H. & Konishi, S. *Brain Res.* **24**, 368-371 (1970).
6. Johnston, G. A. R., Curtis, D. R., Davies, J. & McCulloch, R. M. *Nature* **248**, 804-805 (1974).
7. Biscoe, T. J., Evans, R. H., Headley, P. M., Martin, M. & Watkins, J. C. *Nature* **255**, 166-167 (1975).
8. Olney, J. W., Rhee, V. & Ho, O. L. *Brain Res.* **77**, 507-512 (1974).
9. Coyle, J. T. & Schwarcz, R. *Nature* **263**, 244-246 (1976).
10. McGeer, E. G. & McGeer, P. L. *Nature* **263**, 517-519 (1976).
11. Schwarcz, R. & Coyle, J. T. *Brain Res.* **127**, 235-249 (1977).
12. Elliott, K. A. C. in *Handbook of Neurochemistry* 2 (ed. Lajtha, A.) 103-114 (Vol. 2 Plenum, New York 1966).
13. Hjorth-Simonsen, A. *Stain Tech.* **45**, 199-204 (1970).
14. Hattori, T. & McGeer, E. G. *Brain Res.* **129**, 174-180 (1977).
15. Olney, J. W., Sharpe, L. G. & de Gubareff, T. *Neurosci. Abst.* **1**, 371 (1975).
16. Herkenham, M. *Neurosci. Abst.* **2**, 387 (1976).
17. Meldrum, B. S. & Brierley, J. B. *Brain Res.* **48**, 361-365 (1972).
18. Meldrum, B. S. & Brierley, J. B. *Archs Neurol.* **28**, 10-17 (1973).
19. Meldrum, B. S., Horton, R. W. & Brierley, J. B. *Brain* **97**, 407-418 (1974).
20. Purpura, D. P. & Gonzalez-Montagudo, O. J. *Neuropath. exp. Neurol.* **19**, 421-432 (1960).
21. Nadler, J. V., Vaca, K. W., White, W. F., Lynch, G. S. & Cotman, C. W. *Nature* **260**, 538-540 (1976).
22. Storm-Mathisen, J. *Brain Res.* **120**, 379-386 (1977).
23. White, W. F., Nadler, J. V., Hamberger, A., Cotman, C. W. & Cummins, J. T. *Nature* **270**, 356-357 (1978).
24. Lorente de Nó, R. J. *Psychol. Neurol., Lpz.* **46**, 113-177 (1934).
25. Shute, C. C. D. & Lewis, P. R. *Nature* **199**, 1160-1164 (1963).
26. Shute, C. C. D. & Lewis, P. R. *Z. Zellforsch.* **69**, 334-343 (1966).
27. Haug, F.-M. S. *Z. Anat. Entwickl.-Gesch.* **145**, 1-27 (1974).

***In vitro* release of Leu- and Met-enkephalin from the corpus striatum**

WE have suggested previously that Met-enkephalin and Leu-enkephalin¹ may act as neurotransmitters in the central and peripheral nervous systems^{2,3}. Recent studies on the distribution of the enkephalins support this view⁴⁻⁶. An important step in establishing a possible neurotransmitter role of the enkephalins is the demonstration of their release from neuronal sites by potassium ions or veratridine. Indirect evidence for the release of enkephalin or endorphin has been provided by several workers⁷⁻¹⁰. We now present direct evidence for such release from isolated brain slices and synaptosomal preparations.

The experiments were carried out on preparations of the rabbit and guinea pig striatum, which contain very high endogenous levels of Met- and Leu-enkephalin. The enkephalins were isolated by adsorption on Amberlite CG-400 and elution with formic acid¹¹, followed by adsorption on, and elution with methanol from, Amberlite XAD-2 (ref. 3). All the samples were assayed on the mouse vas deferens with Met- and Leu-enkephalin as standards. Antagonism by naloxone (900 nM) was routinely used in each assay to check for specific opioid peptide activity.

A small basal release of enkephalin (0.4% of tissue content in 30 min) was detected in the superfusate from a layer of crude synaptosomes prepared from rabbit striatum (Table 1). When the potassium ion concentration of the superfusion fluid was increased from 5.94 to 50 mM, the amount of enkephalin recovered in a 15-min collection increased to 1.9% of the tissue content. A second perfusion, 30 min after the first, with Krebs solution containing 50 mM K⁺, produced a much smaller release of enkephalin. In a separate set of experiments, the concentration of calcium chloride was increased to 5.08 mM and the potassium-evoked release of enkephalin was more than double that in Krebs solution containing 2.54 mM calcium chloride. Omission of calcium chloride from the medium mar-

Table 1 Enkephalin release from rabbit striatal synaptosomes

Calcium chloride and potassium ion concentrations in Krebs solution (mM)		No. of experiments	Fractional release (%) during a collection period	
CaCl ₂	K ⁺		1st collection	2nd collection
2.54	5.94	11	0.4±0.2	0.2±0.15
5.08	5.94	4	0.5±0.2	—
2.54	50	4	1.9±0.3	0.5±0.15
5.08	50	4	4.4±0.2	0.9±0.2
0	50	3	0.8±0.1	—
0+EDTA (0.5 mM)	50	3	not detectable	—

A crude synaptosomal fraction (P₂) of rabbit striatum was prepared by homogenising (Teflon pestle and glass tube) 0.8–1.0 g of tissue in 0.32 M sucrose (1 g tissue per 10 ml sucrose). The nuclear fraction (10,000g min) was discarded and the P₂ fraction obtained by centrifuging the supernatant at 17,000g for 20 min. The P₂ pellet was resuspended in Krebs solution and layered on a 1-cm diameter filter superfused with Krebs solution at 36 °C, gassed with 95% O₂ and 5% CO₂. The preparation was superfused for 20 min before collecting the superfusate. Basal release at 2.54 mM CaCl₂ and 5.94 K⁺ was determined in 30-min periods and potassium-evoked release in 15-min collection periods. Fractional release refers to the total enkephalin activity (measured as Met-enkephalin) released during a collection period into the superfusate and is expressed as % of the tissue content. In some experiments a second collection of superfusate was made 30 min after the first collection. All determinations were made in separate experiments, apart from the second collections. The limit of sensitivity of the assay was 0.5 ng. The values are the means±s.e.m.

edly reduced the evoked release of enkephalin; the addition of EDTA to the calcium-free medium reduced the release to undetectable levels.

It has been suggested that Met-enkephalin is merely a proteolytic breakdown product of β -endorphin¹². Experiments were carried out to test the possibility that the source of the released enkephalin might be the larger endorphins released into the medium and subsequently degraded, rather than the direct release of enkephalin. In three perfused synaptosomal preparations, β -endorphin was added to the superfusion medium so that 1 nmol of the peptide passed through the layer of synaptosomes in 15 min. There was no detectable increase in enkephalin above basal levels in each of these experiments. The breakdown of only 0.1% of the β -endorphin to Met-enkephalin would have been detected (sensitivity of assay, 0.5 ng Met-enkephalin), and so we conclude that cleavage of β -endorphin does not occur to any significant extent under our experimental conditions.

The effect of potassium as a depolarising agent is not specific for neurones. Veratridine may be considered a more specific stimulus¹³, and therefore we tested the effect of this agent on enkephalin release from superfused slices of the guinea pig striatum. Preliminary experiments established that the release by veratridine was small and variable, which was considered to be due to enzymatic breakdown of enkephalin. A series of di- and tripeptides were screened for their ability to reduce breakdown. It was found that a combination of leucyl-leucine, leucyl-glycine and tyrosyl-tyrosine (each 1 mM) prevented the breakdown of both Met- and Leu-enkephalin when incubated with either brain homogenates or striatal slices. The inhibition by the dipeptides decreased with time, suggesting

that the mechanism of action might be substrate competition. Control experiments showed that the recovery of added Met-enkephalin from superfused striatal slices increased from 47±5% (*n* = 5) in the absence of the dipeptides to 97±11% (*n* = 5) in their presence.

In the presence of the dipeptides, no basal release of enkephalin (<0.5 ng) could be detected in 10-min collections of striatal superfusate. The addition of veratridine (50 μ M) to the superfusing fluid for 2–10 min caused a release of enkephalin. During a prolonged exposure to veratridine (10 min), the output of enkephalin increased over the first 6 min and then declined (Fig. 1a). When slices were exposed to veratridine for 3 min, at intervals of 30 min, the output of enkephalin declined with successive exposures (Fig. 1b). A similar phenomenon was seen with potassium-evoked enkephalin release from synaptosomes. This decrease in output with successive exposures to veratridine may be due to incomplete recovery of the tissue from the preceding intense stimulation, depletion of the releasable store of enkephalin, or a decrease in the viability of the slices during the course of the experiment. Tetrodotoxin (1.7 μ M) abolished the veratridine-induced output of enkephalin (Fig. 1b).

In another series of experiments, the material released by veratridine was analysed by thin layer chromatography to separate Met- and Leu-enkephalin (Table 2). Both peptides were found to be present in the superfusate and the tissues. The ratio of Met-enkephalin to Leu-enkephalin released from the guinea pig striatum was very similar to the ratio of these peptides present in the tissue.

Our results from striatal synaptosomes and slices indicate that the enkephalins, in common with other putative neurotransmitters, are released from brain tissue by an increase in

Table 2 Comparison of stimulated output and tissue content of Met-enkephalin and Leu-enkephalin

	No. of experiments	Met-enkephalin (ng)*	Leu-enkephalin (ng)*	ratio Met/Leu†
stimulated output	6	5.5±0.9	1.7±0.16	3.4±0.5
tissue content	4	79±22	20±5	3.8±0.2

Guinea pig striatal slices (600- μ m thick, sagittal section) from two striata weighing approximately 300 mg were used in each experiment. The slices were superfused (1.1 ml min⁻¹) for 45 min with Krebs solution at 36 °C and gassed with 95% O₂ and 5% CO₂. The slices were exposed to veratridine (50 μ M) for 10 min and the superfusate collected for 20 min from the start of exposure to veratridine. The dipeptides, Leu-Leu, Tyr-Tyr and Leu-Gly (each 1 mM) were present in the superfusing fluid for 5 min before exposure to veratridine and throughout the collection period. For the determination of endogenous enkephalins, the tissues were homogenised in 0.1 M HCl. All samples (superfusate and tissues) were chromatographed on Amberlite CG-400 and XAD-2 columns, then spotted on 0.25-mm silica gel plates and developed with ethyl acetate:pyridine:water:acetic acid:ethane-1,2-dithiol (100:44:25:11:0.2). The marker lanes were stained with cadmium-ninhydrin reagent, and the areas corresponding to Met-enkephalin (*R*_f $\approx$ 0.4) and Leu-enkephalin (*R*_f $\approx$ 0.55) were removed, eluted and assayed. The values are the means±s.e.m.

*Uncorrected for recovery from all chromatographic procedures; this was estimated to be 30–35%.

†Ratios calculated from individual experiments.

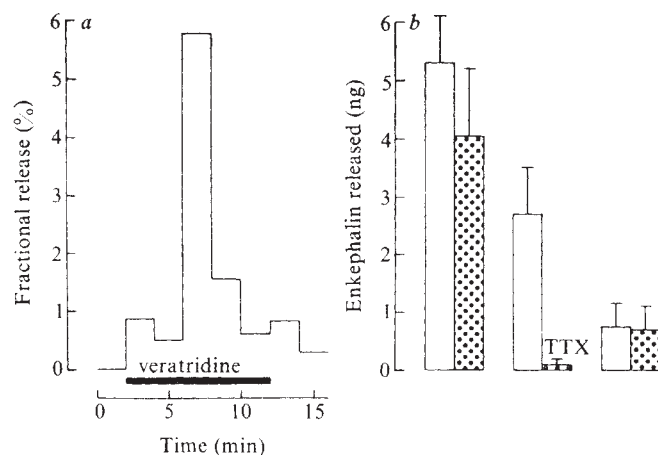


Fig. 1 Release of enkephalin from guinea pig striatal slices. The slices were superfused (1.1 ml per min) for 45 min with Krebs solution at 36 °C and gassed with 95% O₂ and 5% CO₂. *a*, 600 µm thick sagittal sections from two striata weighing approximately 300 mg were exposed to veratridine (50 µM) for 10 min. The superfusate was collected at 2-min intervals. Fractional release refers to the total enkephalin activity (assayed as Met-enkephalin) released into the superfusate expressed as a % of the tissue content. *b*, 450 µm thick sagittal sections from one striatum weighing approximately 150 mg were exposed to veratridine (50 µM) for 3 min every 30 min. The superfusate was collected for 12 min from the start of veratridine exposure. The open columns represent the output of enkephalin on three successive exposures to veratridine. The stippled columns represent the output of enkephalin from tissues in which the second exposure to veratridine was made in the presence of 1.6 µM tetrodotoxin (TTX). All outputs were assayed as Met-enkephalin. Each column represents the means from three experiments, the vertical bar indicating s.e.m. In both *a* and *b*, Leu-Leu, Tyr-Tyr and Leu-Gly (each 1 mM) were present in the superfusing fluid for 5 min before exposure to veratridine and throughout the collection periods. All samples (superfusate and tissues) were chromatographed on only Amberlite CG-400 and XAD-2 columns with a recovery of 90–100%. No attempt was made to separate Leu-enkephalin from Met-enkephalin.

potassium ions or veratridine. The potassium-induced release is calcium-dependent and the effect of veratridine is abolished by tetrodotoxin. We suggest that the source of this evoked release is the neuronal stores of enkephalins and that the present results provide additional direct evidence that Met- and Leu-enkephalin may act as central neurotransmitter agents.

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G. HENDERSON*
J. HUGHES†
H. W. KOSTERLITZ

Unit for Research on Addictive Drugs,
University of Aberdeen,
Marischal College,
Aberdeen, UK

Received 31 October 1977; accepted 9 January 1978.

Present addresses: * Loyola University Stritch School of Medicine, Department of Pharmacology, Maywood, Illinois 60153; † Imperial College of Science and Technology, Department of Biochemistry, South Kensington, London, SW7, UK.

- Hughes, J. *et al.* *Nature* **258**, 577–579 (1975).
- Kosterlitz, H. W. & Hughes, J. *Life Sci.* **17**, 91–96 (1975).
- Hughes, J., Kosterlitz, H. W. & Smith, T. W. *Br. J. Pharmacol.* **61**, 639–647 (1977).
- Elde, R. P., Hokfelt, T., Johansson, O. & Terenius, L. *Neuroscience* **1**, 349–351 (1976).
- Hokfelt, T. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **74**, 3081–3085 (1977).
- Simantov, R., Kuhar, M. J., Uhl, G. R. & Snyder, S. H. *Proc. natn. Acad. Sci. U.S.A.* **74**, 2167–2171 (1977).
- Waterfield, A. A. & Kosterlitz, H. W. *Life Sci.* **16**, 1787–1792 (1975).
- Jacob, J. J., Tremblay, E. C. & Colombeau, M. C. *Psychopharmacologia* **37**, 217–223 (1974).
- Van Neuten, J. M., Janssen, P. A. J. & Fontaine, J. *Life Sci.* **18**, 803–808 (1976).
- Puig, M. M., Gascon, P., Gravisio, G. L. & Musacchio, J. M. *Science* **195**, 419–420 (1977).
- Hughes, J. *Brain Res.* **88**, 295–306 (1975).
- Smyth, D. G. & Snell, C. R. *FEBS Lett.* **78**, 225–228 (1977).
- Benfiorado, J. M. in *Physiological Pharmacology* (eds Root, W. S. & Hoffmann, F. G.) **4**, 331–398 (Academic, New York, 1967).

Release of enkephalin from rat globus pallidus *in vitro*

SINCE the discovery of the endogenous opioid peptides Leu- and Met-enkephalin in mammalian brain, there has been considerable interest in their possible physiological functions^{1–3}. Recent immunohistochemical studies have shown that the enkephalins are localised in neurones in various regions of the central nervous system, with high concentrations in nerve terminals^{4,5}. These morphological studies and the results of detailed regional analyses of rat brain by radioimmunoassay have indicated that enkephalin-containing nerve terminals are particularly concentrated in the rat globus pallidus, which by radioimmunoassay has been found to contain a concentration of enkephalin six to eight times higher than that observed in any other brain region^{6,7}. Here, we describe a calcium-dependent evoked release of enkephalin-like immunoreactivity from slices of rat globus pallidus *in vitro*. This finding supports the view that enkephalins may be released as neurotransmitter substances from nerve terminals in the brain.

Globus pallidus tissue was dissected from each side of thick frontal sections of chilled rat brain, using the anatomical landmarks of the atlas of König and Klippel⁸. The pooled samples of tissue from left and right sides weighed approximately 30 mg and were contaminated with small amounts of adjacent striatal and thalamic tissue. The tissue from each brain was cross-cut at 200-µm intervals with a McIlwain tissue chopper, and the resulting slices were suspended in Krebs-bicarbonate medium in a small plastic superfusion chamber at 37 °C, as described previously^{9,10}. The slices were superfused with a modified Krebs-bicarbonate medium at 37 °C, containing bovine serum albumin and bacitracin to protect against loss of released enkephalin by absorption or metabolism. After an initial wash period of 13 min, superfusate samples were collected at 3-min intervals and aliquots were analysed for enkephalin using a sensitive radioimmunoassay procedure^{11,12}. This assay involved the use of an anti-Leu-enkephalin rabbit serum for which Leu-enkephalin had an affinity some 30 times higher than that of Met-enkephalin. It was thus not possible to distinguish between Leu- and Met-enkephalin, and the results were calculated in terms of units of enkephalin (1 unit = 1 ng Leu-enkephalin equivalent). Since rat globus pallidus is reported to contain a sixfold preponderance of Met-enkephalin over Leu-enkephalin⁷, the observed immunoreactivity was probably due to a mixture of the two peptides.

A rapid release of enkephalin-like immunoreactivity was observed during the initial 13-min wash-out period; after this, however, the spontaneous release of enkephalin decreased to 0.1–0.2% of the total tissue stores per minute. In several instances, the concentration of enkephalin present in such spontaneous release samples was less than 10 pg ml⁻¹, which represented the lower limit of accurate measurement for the radioimmunoassay procedure. During exposure to a high potassium medium (K⁺ 50 mM) for a 6-min period, there was a large increase in the rate of release of enkephalin-like immunoreactivity (Fig. 1a). During the period of potassium ion stimulation the rate of enkephalin release was 10–20 times higher than in the preceding spontaneous release samples, and a total of 9.6 ± 1.8% of the total tissue enkephalin content was released during the 6-min stimulation period (mean ± s.e.m., *n* = 10 experiments). On return to normal medium the efflux of enkephalin decreased rapidly to resting values similar to those observed before the stimulus. The release of enkephalin was very rapid during the initial 3 min of the period of potassium ion exposure, and was significantly slower during the second 3 min of the potassium ion pulse (Fig. 1). Further evidence for an exhaustion of the tissue stores of releasable enkephalin was obtained from experiments in which the tissue slices were exposed to a second potassium ion pulse 9 min after the first exposure (Fig. 2). The second exposure to potas-

sium ion released less than half as much enkephalin as the first, although the tissue content of enkephalin at the beginning of the second stimulation period was only some 10% lower than at the beginning of the first period of stimulation.

In the absence of calcium in the superfusion medium, the spontaneous release of enkephalin occurred at a low rate, similar to that observed in control experiments, and high potas-

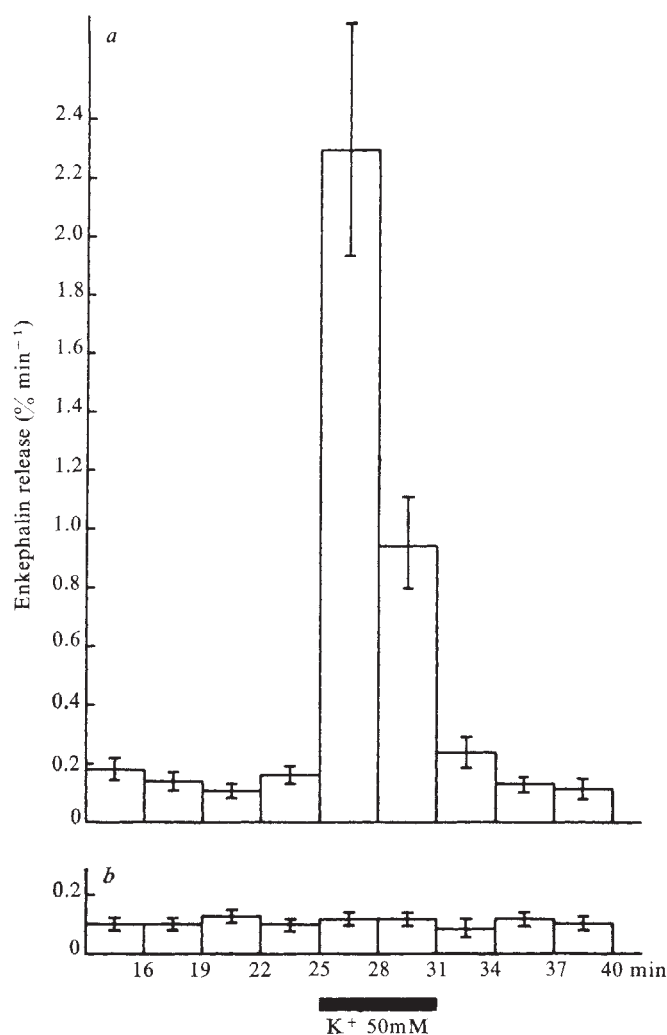


Fig. 1 Release of enkephalin immunoreactivity from slices of rat globus pallidus superfused with normal (a) or calcium-free medium (b). The slices of pallidal tissue (about 30 mg wet weight) were superfused at a rate of approximately 300 $\mu\text{l min}^{-1}$, and after an initial wash period of 13 min, samples of the superfusate were collected at 3-min intervals for subsequent radioimmunoassay. The superfusion medium was a modified Krebs-bicarbonate solution with the following composition: NaCl 127 mM; KCl 3.73 mM; CaCl_2 1.8 mM; KH_2PO_4 1.18 mM; MgSO_4 1.18 mM; NaHCO_3 20 mM; D-glucose 2 g l^{-1} . The medium was gassed with an oxygen/carbon dioxide (95%:5%) mixture, and contained bovine serum albumin (Sigma, crystalline) (0.1%, w/v) and the peptidase inhibitor bacitracin (Sigma) (30 $\mu\text{g ml}^{-1}$). Twenty microlitres of a saturated solution of Na_3EDTA was added to each of the tubes used to collect the superfusate samples as a further precaution to protect released enkephalin from enzymatic degradation. During the period indicated by the horizontal black bars the slices were exposed to a medium in which KCl was substituted for NaCl to give a final K^+ concentration of 50 mM. One hundred microlitre aliquots of the superfusates were analysed for enkephalin using a radioimmunoassay procedure¹¹. Results are expressed as percentage total tissue enkephalin released per minute, based on measurements of the total tissue enkephalin content at the end of the superfusion (Table 1). This was estimated after extraction of the tissue with boiling 1 M acetic acid, homogenisation and neutralisation of the centrifuged extracts, as described previously¹¹. Control values (a) represent means $\pm$ s.e.m. for 10 experiments; results obtained with calcium-free medium (b) are means $\pm$ s.e.m. for four experiments.

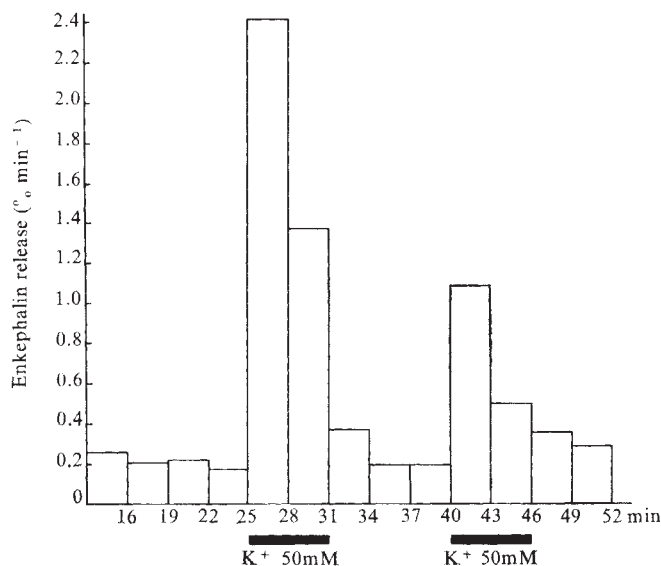


Fig. 2 Release of enkephalin from rat globus pallidus slices in response to two periods of exposure to high potassium medium. Mean values for two experiments; conditions as in Fig. 1a.

sium ion exposure was completely without effect in stimulating enkephalin release (Fig. 1b).

Measurements of the total enkephalin-like immunoreactivity in extracts of freshly dissected globus pallidus samples (Table 1) confirmed that this area of rat brain contains a very high concentration of enkephalin^{6,7}, amounting to 377 ± 15.4 units per g wet weight (mean $\pm$ s.e.m., $n = 4$), a value some three to four times higher than that observed in rat striatum or hypothalamus in a previous regional survey using the same radioimmunoassay procedure¹². After a 40-min period of superfusion *in vitro*, the total enkephalin immunoreactivity of the tissue slices was markedly reduced when compared with the values obtained from fresh samples of globus pallidus tissue, indicating that some 60% of the total tissue enkephalin content was lost during the course of the experiments (Table 1). This is in contrast to the results obtained in analogous experiments with the other neuropeptides, substance P^{6,16} or somatostatin and neurotensin (L.L.L., S.D.L., F.E.B., M. Brown and W. Vale, unpublished), in which only a small proportion of the tissue stores of peptides were lost during a similar period of superfusion. The loss of tissue enkephalin during superfusion could not all be accounted for by the amounts of free peptide measured in the superfusate samples. During the 40-min superfusion period, approximately 25% of the total tissue enkephalin content was recovered as immunoreactive material in the superfusate samples; the remaining substantial loss of tissue enkephalin must presumably be attributed to enzymatic degradation, which was only partially inhibited by the addition of bacitracin¹³. The importance of adding this peptidase inhibitor was illustrated by the results of experiments in which bacitracin was

Table 1 Tissue content of enkephalin immunoreactivity in rat globus pallidus

Conditions	No. of experiments	Tissue enkephalin (units per globus pallidus sample)
Freshly dissected	4	11.32 ± 0.46
At end of 40-min control superfusion	10	4.62 ± 0.38
At end of 40-min superfusion in calcium-free medium	4	4.06 ± 0.52
At end of 40-min superfusion with control medium without bacitracin	4	4.17 ± 0.53

omitted. In these conditions, the spontaneous release of enkephalin was so low that it could not be measured accurately by the radioimmunoassay procedure in a majority of the resting efflux samples. Furthermore, although exposure to high potassium ion evoked a measurable release of enkephalin, the amounts collected during the 6-min period of exposure to high potassium ion represented only $5.8 \pm 1.5\%$ of the total tissue stores (mean $\pm$ s.e.m., $n = 4$), significantly lower than the values observed in response to high potassium ion in media containing bacitracin. The tissue content of enkephalin at the end of these experiments, however, was not significantly different, whether or not bacitracin had been added to the medium (Table 1), suggesting that protection from enzymatic degradation is not important for stabilising enkephalin in its intracellular storage sites, but is critical in inhibiting degradation after the peptide has been released.

Because of this degradation, the observed percentage of enkephalin released should be viewed with some reservation. Since our antisera read both enkephalins, more detailed analysis of the release and stability of each enkephalin in these experimental conditions will be needed in order to arrive at more accurate estimates of the percentage of the total enkephalin content which can be released by potassium *in vitro*. Preliminary experiments indicate that the rate of catabolism of Met-enkephalin within the perfusion chambers is faster than that of Leu-enkephalin⁵. Nevertheless, the overall results of the present experiments have been validated in a variety of experimental conditions, with controls for release and peptidase inhibition.

In summary, the present results show that enkephalin can be released from rat brain *in vitro* in response to depolarisation caused by elevation of the external potassium ion concentration, and that this evoked release is completely dependent on the presence of calcium ions in the external medium. Such behaviour is characteristic of a number of other substances thought to be released as neurotransmitters in the central nervous system¹⁴⁻¹⁶, and strongly supports a neurotransmitter role for enkephalin in mammalian brain. A similar calcium-dependent potassium-evoked release of enkephalin has been described from rat brain synaptosome preparations¹⁷ and from rabbit and guinea pig striatal tissue¹⁸.

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L. L. IVERSEN*
S. D. IVERSEN†
F. E. BLOOM
T. VARGO
R. GUILLEMIN

A. V. Davis Center for Behavioral Neurobiology,
and the Neuroendocrinology Laboratory,
The Salk Institute, La Jolla, California

*Permanent address: *MRC Neurochemical Pharmacology Unit, Department of Pharmacology, University of Cambridge, UK; †Department of Experimental Psychology, University of Cambridge, UK.

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- Hughes, J. *et al.* *Nature* **258**, 577 (1975).
- Simantov, R. & Snyder, S. H. *Life Sci.* **18**, 781 (1976).
- Kosterlitz, H. W. (ed) *Opiates and Endogenous Opioid Peptides* (North-Holland, Amsterdam, 1976).
- Elde, R., Hokfelt, T., Johansson, D. & Terenius, L. *Neuroscience* **1**, 349 (1977).
- Simantov, R., Kuhar, M. J., Uhl, G. R. & Snyder, S. H. *Proc. natn. Acad. Sci. U.S.A.* **74**, 2167 (1977).
- Hong, J. S., Yang, H. Y. T., Fratta, W. & Costa, E. *Brain Res.* **134**, 383 (1977).
- Kobayashi, R. M., Palkovits, M., Miller, R. T., Chang, K. J. & Cuatrecasas, P. *Life Sci.* (in the press).
- König, J. F. R. & Klippel, R. A. *The Rat Brain* (R. E. Krieger, Huntington, New York, 1967).
- Jessell, T. M., Iversen, L. L. & Kanazawa, I. *Nature* **264**, 81 (1976).
- Jessell, T. M. & Iversen, L. L. *Nature* **268**, 549 (1977).
- Rossier, J. *et al.* *Life Sci.* **21**, 847-852 (1977).
- Rossier, J. *et al.* *Proc. natn. Acad. Sci. U.S.A.* (in the press).
- Miller, R. J., Chang, K. J. & Cuatrecasas, P. *Biochem. biophys. Res. Commun.* **74**, 1311 (1977).
- Geffen, L. B., Jessell, T. M., Cuello, A. C. & Iversen, L. L. *Nature* **260**, 258 (1976).
- Mulder, A. H. & Snyder, S. H. *Brain Res.* **76**, 297 (1974).
- Schenker, C., Mroz, E. A. & Leeman, S. E. *Nature* **264**, 790 (1976).
- Smith, T. W., Hughes, J., Kosterlitz, H. W. & Sosa, R. P. in *Opiates and Endogenous Opioid Peptides* (ed. Kosterlitz, H. W.) 57-62 (North Holland, Amsterdam, 1976).
- Henderson, G., Hughes, J. & Kosterlitz, H. W. *Nature* **271**, 677-679 (1978).

Inhibition of hepatitis B DNA polymerase by intercalating agents

INFECTION with hepatitis B virus (HBV) is widespread and increasing in incidence in many countries. Unfortunately, there is as yet no effective chemotherapy. The hepatitis B virion is unique in that it contains both circular DNA and a DNA polymerase enzyme (for review see ref. 1). After the description of the enzyme² in concentrated preparations of hepatitis B antigen (HBsAg) containing Dane particles it was shown that enzyme activity was inhibited by intercalating agents such as ethidium bromide³. We have extended these early studies to include intercalating agents that can be safely administered to patients. We show here that several intercalating agents in clinical use inhibit the hepatitis B (HB) DNA polymerase reaction *in vitro* and we discuss the implications of these findings for the therapy of hepatitis B infection.

The following compounds were included in the study: ethidium bromide (molecular weight 394.3), chloroquine diphosphate (MW 515.9), primaquine diphosphate (MW 455.4), quinacrine hydrochloride (MW 472.9), methylene blue (MW 319.9), chlorpromazine (MW 355.3), quinine hydrochloride (MW 360.9) (all from Sigma) and hydroxychloroquine (MW 530, Sterling-Winthrop). Ethidium bromide and chloroquine have been shown to intercalate by changes induced in the superhelical density of the replicative form of Φ X174 DNA (ref. 4). Quinacrine, chlorpromazine, methylene blue and quinine change the superhelical density of PM-2 DNA in the manner of intercalators when examined by viscometric titration⁵. Primaquine has been shown to change the superhelical density of PM-2 DNA in the manner of intercalators at low ionic strength⁵.

Pellets of HBsAg containing Dane particles were prepared from patients' sera and shown to contain hepatitis B surface antigen (HBsAg) and hepatitis B core antigen (HBcAg) by methods described previously⁶. Assays for HB DNA polymerase were carried out in reaction mixture containing 0.15 M KCl and 0.01 M $MgCl_2$ (ref. 7), and in the differential assay mix⁸ containing 0.4 M KCl and 0.04 M $MgCl_2$ as previously described^{2,6,7}. The DNA product of the reaction was shown to be associated with HBcAg (ref. 6). Concentrated stock solutions of the drugs were prepared in distilled water and aliquots were added to the reaction mixtures to give the desired concentrations. The concentration of intercalating agent resulting in 50% inhibition (IC_{50}) of the HB DNA polymerase reaction was calculated from the results by a modification of the method of Reed and Muench⁹ where applicable.

Ethidium bromide, the archetype intercalating agent, was the most effective inhibitor of hepatitis B DNA polymerase amongst the drugs examined. The DNA polymerase reaction was inhibited by 90% at a concentration of $10 \mu g\ ml^{-1}$ ($2.5 \times 10^{-5} M$) of ethidium bromide in reaction mixture containing 0.15 M KCl and 0.01 M $MgCl_2$ (Fig. 1a); the IC_{50} was $3.2 \mu g\ ml^{-1}$ ($8.1 \times 10^{-6} M$). Higher concentrations of magnesium ion interfered with inhibitory effect of ethidium bromide. Thus in 0.04 M $MgCl_2$ only 77% of the DNA polymerase reaction was inhibited at a concentration of $25 \mu g\ ml^{-1}$ of ethidium bromide and the IC_{50} was $10 \mu g\ ml^{-1}$ (Fig. 1a). Inclusion of the non-ionic detergent Nonidet P-40 (NP-40) in the reaction mixture did not affect the inhibitory action of ethidium bromide on HB DNA polymerase.

The other intercalators studied, although not as toxic as ethidium bromide, are much weaker intercalators and were weaker inhibitors of HB DNA polymerase. However, several of the drugs inhibited HB DNA polymerase substantially at concentrations achievable in human liver⁹. Chloroquine, the mainstay of malarial prophylaxis, inhibited the DNA polymerase reaction by 45 and 53% at concentrations of 250 and $500 \mu g\ ml^{-1}$ respectively (Fig. 1b); the IC_{50} was $480 \mu g\ ml^{-1}$ ($9.30 \times 10^{-4} M$; Table 1). Quinacrine was a more effective inhibitor of HB DNA polymerase than chloroquine

(Fig. 1b); the IC_{50} for quinacrine was $334 \mu\text{g ml}^{-1}$ ($7.1 \times 10^{-4}\text{M}$; Table 1). Inclusion of NP-40 in the reaction mixture at a concentration of 0.3% interfered with the inhibition of the DNA polymerase reaction by chloroquine (Fig. 1b); NP-40 also interfered with the inhibition of the enzyme by quinacrine. Magnesium ion reduced the inhibitory activity of chloroquine (Fig. 1b); thus at a concentration of chloroquine of $500 \mu\text{g ml}^{-1}$ only 16% of the reaction was inhibited in the presence of 0.03 M MgCl_2 (Fig. 1b). Primaquine, another antimalarial

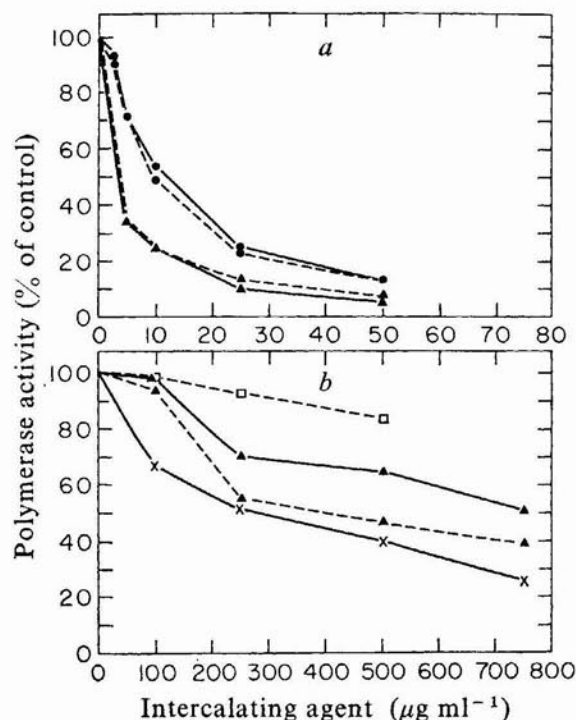


Fig. 1 Inhibition of hepatitis B DNA polymerase by intercalating agents. Pellets containing particulate HBAG, including Dane particles, were prepared by diluting serum 1:2 with phosphate-buffered saline (PBS), and centrifuging at $39,000g$ at 4°C , as previously described^{2,6}. The resulting pellet was washed in an equal volume of PBS or centrifuged through 5–20% sucrose gradients in PBS⁶. The final pellets were taken up in PBS in 1:20 of the original volume of serum. The standard DNA polymerase reaction mixture^{6,7} contained 0.05 M Tris-HCl, 20 mM dithiothreitol (DTT), 1 mM each of dATP, dCTP, and dGTP, and 2.5 μCi of ^3H -dTTP at pH 7.5 in a final total volume of 0.1 ml. KCl, MgCl_2 , and NP-40, were included in the reaction mixture at various concentrations. The complete reaction mixture, including DNA polymerase-containing pellet suspension, was incubated at 38°C for 180 min. Enzyme activity was measured by the incorporation of ^3H -dTTP into acid-insoluble product^{6,7}. The ordinate represents enzyme activity expressed as % of control reactions not containing intercalators and the abscissa represents the concentration of intercalators in $\mu\text{g ml}^{-1}$. The same quantity of polymerase-containing pellet suspension was included in each reaction mixture. **a**, Ethidium bromide: $\bullet$ — $\bullet$, reaction mixture contained 0.4 M KCl, 0.04 M MgCl_2 and 0.3% NP-40; $\bullet$ — $\bullet$, same reaction mixture without NP-40; $\blacktriangle$ — $\blacktriangle$, reaction mixture contained 0.15 M KCl and 0.01 M MgCl_2 with 0.3% NP-40; $\blacktriangle$ — $\blacktriangle$, same reaction mixture without NP-40. Control reaction mixtures without intercalators in 0.04 M MgCl_2 with 0.3% NP-40 incorporated 2,200 c.p.m. of ^3H -dTTP per 0.1 ml reaction mixture into DNA; without NP-40 incorporation was 1,250 c.p.m. Control reactions without intercalators in 0.01 M MgCl_2 with NP-40 incorporated 1,100 c.p.m. of ^3H -dTTP and 875 c.p.m. without NP-40. Incorporation of 100 c.p.m. represents 4×10^{-3} pmol of ^3H -dTTP. **b**, $\blacktriangle$ — $\blacktriangle$, Chloroquine in reaction mixture containing 0.15 M KCl, 0.01 M MgCl_2 and 0.3% NP-40; $\blacktriangle$ — $\blacktriangle$, same reaction mixture with chloroquine but without NP-40. $\square$, Chloroquine in reaction mixture containing 0.15 M KCl, 0.03 M MgCl_2 without NP-40. $\times$, Quinacrine in reaction mixture containing 0.15 M KCl, 0.01 M MgCl_2 without NP-40. Incorporation of ^3H -dTTP in control reactions without intercalators is given in (a) except that control reactions in 0.15 M KCl, 0.03 M MgCl_2 incorporated, 1,048 c.p.m.

agent, was a weak inhibitor of HB DNA polymerase; the IC_{50} of primaquine was $900 \mu\text{g ml}^{-1}$ ($2 \times 10^{-3}\text{M}$; Table 1). On the other hand, chlorpromazine was a very active inhibitor of the DNA polymerase reaction with an IC_{50} of $250 \mu\text{g ml}^{-1}$ ($7 \times 10^{-4}\text{M}$). Hydroxychloroquine and quinine hardly inhibited the enzyme reaction. However, methylene blue was a potent inhibitor of HB DNA polymerase; 94% of the reaction was inhibited at a concentration of $250 \mu\text{g ml}^{-1}$ of methylene blue ($7.8 \times 10^{-4}\text{M}$).

The inhibitory activity of combinations of intercalating agents against HB DNA polymerase was examined in order to determine whether additive or even synergistic effects would be observed. The combination of chloroquine and primaquine yielded moderately additive inhibitory effects on HB DNA polymerase (Table 2). Thus the combination of chloroquine at $500 \mu\text{g ml}^{-1}$ with primaquine at $250 \mu\text{g ml}^{-1}$ inhibited the DNA polymerase reaction by 66%, whereas at these concentrations each drug alone inhibited the reaction by 50 and 15%, respectively. However, the combination of chloroquine and primaquine at $500 \mu\text{g ml}^{-1}$ for each drug was not much more inhibitory than the lower concentrations (Table 2). The combination of chloroquine and quinacrine also showed moderately additive inhibitory activity against HB DNA polymerase. The combination of $500 \mu\text{g ml}^{-1}$ of chloroquine with 250 and $500 \mu\text{g ml}^{-1}$ quinacrine inhibited the DNA polymerase reaction by 71 and 78%, respectively (Table 2). In general the additive inhibitory effect of chloroquine combined with either primaquine or quinacrine was not much greater than the inhibition shown by the more active agent alone. The combination of chloroquine and chlorpromazine was quite effective in inhibiting the DNA polymerase reaction: $500 \mu\text{g ml}^{-1}$ of each drug combined inhibited the enzyme by 81% (Table 2). Chloroquine with methylene blue was an extremely potent inhibitory combination against HB DNA polymerase. Chloroquine at $500 \mu\text{g ml}^{-1}$ combined with 250 and $500 \mu\text{g ml}^{-1}$ of methylene blue inhibited the DNA polymerase reaction by 95 and 99.7%, respectively (Table 2). Primaquine combined with quinacrine also showed additive inhibitory effects against HB DNA polymerase (Table 2). The combination of quinacrine and chlorpromazine was extremely potent in inhibiting the DNA polymerase reaction; quinacrine $500 \mu\text{g ml}^{-1}$ combined with $100 \mu\text{g ml}^{-1}$ chlorpromazine inhibited the reaction by 84% (Table 2). Thus although the combinations of intercalators examined did not show synergistic effects, additive inhibitory effects were shown for all the drug combinations examined.

Of the three major antimalarial drugs examined primaquine was a much weaker inhibitor of HB DNA polymerase than either chloroquine or quinacrine. Primaquine seems to act as a preferential inhibitor of protein biosynthesis by causing a rapid breakdown of ribosomes when studied *in vivo*^{10,11}. Primaquine at $6 \times 10^{-4}\text{M}$ inhibits protein synthesis almost completely in *Bacillus megaterium* but has only marginal effects on the biosynthesis of DNA and ribonucleic acid¹⁰. The two phenothiazine derivatives, chlorpromazine and methylene blue, were quite active in inhibiting HB DNA polymerase. Careful study of the binding of these two compounds to DNA, in conditions selected to avoid precipitation of the DNA by the phenothiazine derivatives, by Allison and Hahn⁵ showed that these two drugs

Table 1 Comparison of inhibitory activity of intercalators against hepatitis B DNA polymerase

Intercalator	IC_{50} ($\mu\text{g ml}^{-1}$)
Ethidium bromide	3.2
Chloroquine	480
Quinacrine	334
Primaquine	900
Chlorpromazine	250

Reaction mixtures contained 0.15 M KCl and 0.01 M MgCl_2 without NP-40. Experimental details are given in the text and legend to Fig. 1.

Table 2 Inhibition of hepatitis B DNA polymerase by combinations of intercalators

CQ	Combinations of intercalators ($\mu\text{g ml}^{-1}$)				Enzyme activity (% of control)
	PQ	QC	CP	MB	
750	100	—	—	—	33
500	100	—	—	—	47
500	250	—	—	—	34
500	500	—	—	—	31
250	100	—	—	—	67
250	250	—	—	—	60
100	100	—	—	—	73
500	—	100	—	—	37
500	—	250	—	—	29
500	—	500	—	—	22
250	—	250	—	—	46
500	—	—	100	—	31
500	—	—	250	—	28
500	—	—	500	—	19
500	—	—	—	250	5
500	—	—	—	500	0.3
—	500	250	—	—	38
—	500	500	—	—	28
—	—	500	100	—	16
—	—	500	250	—	10

Reaction mixtures contained 0.15 M KCl and 0.01 M MgCl_2 without NP-40. Reaction mixtures included the combination of drugs at the concentrations shown. Other experimental details as detailed in the text and in the legend to Fig. 1. CQ, chloroquine; PQ, primaquine; QC, quinacrine; CP, chlorpromazine; MB, methylene blue.

did bind to DNA in the manner of intercalators. Interestingly, neither quinine nor hydroxychloroquine were active inhibitors of the DNA polymerase. The observation that the various combinations of chloroquine, primaquine and quinacrine were only moderately more inhibitory than the more active drug in the combination suggested that the drugs all attach to the same site on the DNA. Inhibition of HB DNA polymerase by the intercalators decreased as the concentration of MgCl_2 was increased. Magnesium interferes with the binding of intercalators to DNA⁴.

Our results show that hepatitis B DNA polymerase can be inhibited by drugs in clinical use. It has been suggested that the DNA polymerase of the hepatitis B virion plays an important part in the replicative cycle of this virus in the hepatocyte¹. Thus, our findings suggest a new approach to the therapy of HBV infection using intercalating agents either singly or in combination. Drugs such as chloroquine and quinacrine accumulate in very high concentrations in the human liver during therapy⁹, and this pharmacologic property may make it feasible to treat HBV infection, even though high concentrations are required to inhibit HB DNA polymerase. Furthermore, the possible therapeutic effects of these template blocking agents may be examined in combination with DNA synthesis inhibitors or with interferon¹². Interestingly, intercalating agents have the singular property of eliminating bacterial plasmids^{13,14}. Thus it may be speculated that these compounds may remove and thus 'cure' the infected hepatocyte from HBV DNA. Clinical studies to evaluate the therapeutic possibilities of intercalating agents in human HBV infection are under way.

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SHALOM Z. HIRSCHMAN
ESTHER GARFINKEL

Division of Infectious Diseases,
Department of Medicine,
Mount Sinai School of Medicine,
New York, New York 10029

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1. Hirschman, S. Z. *Lancet* ii, 436-438 (1975).
2. Hirschman, S. A., Vernace, S. & Schaffner, F. *Lancet* ii, 1099-1103 (1971).
3. Hirschman, S. Z. *Trans. N.Y. Acad. Sci.* 33, 595-606 (1971).
4. Waring, M. J. *molec. Biol.* 54, 247-279 (1970).
5. Allison, R. G. & Hahn, F. E. *Antimicrob. Agents Chemother.* 11, 251-257 (1977).
6. Hirschman S. Z. & Garfinkel, E. J. *infect. Dis.* 135, 897-910 (1977).

7. Hirschman, S. Z., Gerber, M. & Garfinkel, E. *Proc. natn. Acad. Sci. U.S.A.* 71, 3345-3349 (1974).
8. Reed, L. J. & Meunch, H. *Am. J. Hyg.* 27, 493-498 (1938).
9. Goodman, L. S. & Gilman, A. *The Pharmacologic Basis of Therapeutics* (Macmillan, New York, 1975).
10. Olenick, J. G. & Hahn, F. E. *Antimicrob. Agents Chemother.* 1, 259-262 (1972).
11. Olenick, J. G. *Antimicrob. Agents Chemother.* 8, 754-756 (1975).
12. Greenberg, H. B. *et al. New Engl. J. Med.* 295, 517-522 (1976).
13. Hahn, F. E. & Ciak, J. *Ann. N.Y. Acad. Sci.* 182, 295-304 (1971).
14. Hahn, F. E. & Ciak, J. *Antimicrob. Agents Chemother.* 9, 77-80 (1976).

Relationship between β converting and γ non-converting corynebacteriophage DNA

BACTERIOPHAGE can convert non-toxin producing strains of *Corynebacterium diphtheriae* to toxinogeny, and the significance of the difference between β -tox⁺ converting corynebacteriophage isolated by Freeman¹ and γ -tox non-converting phage isolated in this laboratory² has been studied sporadically over the past 20 yr. These two phages are serologically related, but are heteroimmune when tested reciprocally against their lysogenic derivatives³. Both phages exhibit similar one-step growth characteristics and patterns of inducibility by ultraviolet light⁴ and are morphologically similar though differing slightly in head and tail measurements⁵. The phages recombine vegetatively, though at a frequency lower than in homoimmune β matings⁶ and also recombine as prophages in tandem double lysogens⁷. It has been shown⁸ that γ non-converting phage probably carries a cryptic *tox* gene, or part of the gene, as matings between β -tox and γ -tox have yielded *tox*⁺ recombinants. These data all suggest that there is a high degree of genetic homology between these two phages. To assess this point more directly we have examined DNA heteroduplexes of the phages and DNA fragments produced by various restriction endonucleases. The data show that the two phages are almost identical.

Heat-inducible mutants of β and γ phages, β -tsr3 and γ -tsr1, isolated in this laboratory⁹ were used for the production of purified phage DNA. These mutants are unlikely to vary significantly from wild-type phage because both are conditional lethals, presumably a result of point mutations and thus not detectably different from the wild type in heteroduplex analyses. Lysogens of these phages in strain C7 of *C. diphtheriae* were induced in tryptose yeast extract broth¹⁰ supplemented with 0.2% Tween 80. The phage were concentrated directly from lysates by high-speed centrifugation (100,000g) following an initial low-speed cycle (8,000g). The pellets were immediately resuspended in 0.05 M EDTA, 0.1 M NaCl, 0.05 M Tris buffer (pH 7.2) and the DNA was extracted sequentially, twice with equal volumes of chloroform:isoamyl alcohol (24:1). After dialysis against 0.006 M Tris (pH 7.5) the contaminating RNA was eliminated by a treatment for 1 h at 60 °C with ribonuclease (Calbiochem) at 0.05 mg ml⁻¹ in 0.01 M NaCl, 0.0005 M EDTA, 0.002 M Tris (pH 8.0). The purified DNA was then dialysed exhaustively as above. No contaminating bacterial DNA was detected in whole-phage DNA preparations subjected to Agarose gel electrophoresis. Melting profiles also demonstrated the homogeneity of the DNA preparations, and showed that the mole fraction of guanine plus cytosine in both β -tsr3 and γ -tsr1 was 0.54. These data, together with microscopic evidence, show that corynebacteriophage DNA is double stranded.

To determine the molecular mass of the phage genomes, whole phage DNA was prepared and mounted on grids for examination in the electron microscope by the Kleinschmidt technique¹¹. RSF2124, a 7.4×10^6 molecular mass derivative of the *Escherichia coli* plasmid ColE1 with an inserted Tn3 (provided by P. Shipley) was used as a molecular mass standard¹². The contour lengths of 21 linear β -tsr3 molecules were measured and from these a mean molecular mass of

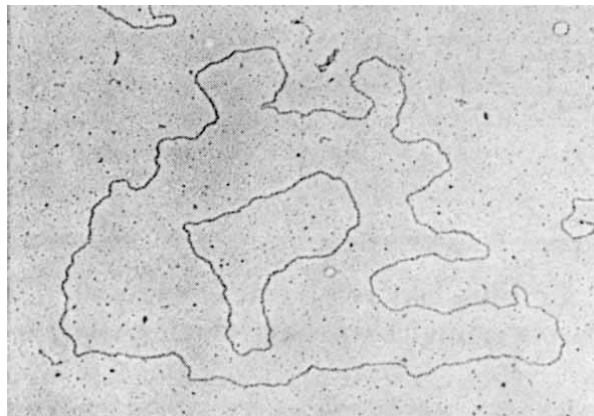


Fig. 1 Electron micrograph of γ -*tsr1* DNA spread for the determination of molecular mass. The small molecular mass standard, RSF2124, is encircled by γ -*tsr1* DNA.

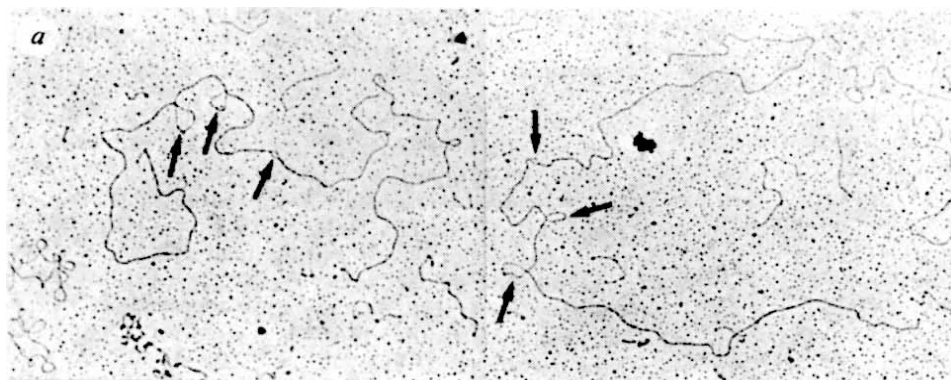
$22.9 \pm 0.31 \times 10^6$ ($\pm$ standard error) was calculated. Similarly, 20 γ -*tsr1* molecules had a mean molecular mass of $25.2 \pm 0.51 \times 10^6$. These data compare favourably to the estimate of 22×10^6 for wild-type β DNA reported by Wolfson and Dressler (quoted in ref. 13). Circular molecules noted in these preparations (Fig. 1) were also measured. The molecular mass as calculated from the contour length of 21 circular β -*tsr3* molecules was $22.9 \pm 0.08 \times 10^6$, and was $24.9 \pm 0.07 \times 10^6$ for 19 circular γ -*tsr1* molecules. The presence of circular molecules in the DNA preparations suggests that packaged β and γ phages have sticky, single-stranded ends similar to the lamboid phages of *E. coli*. Laird and Groman, in their study of the prophage map of β phage⁷, postulated that circularisation of the vegetative phage genome precedes integration into the bacterial genome. Our findings provide physical evidence that this step is possible.

Heteroduplexes of the two corynebacteriophage mutants

were prepared¹⁴ and examined in the electron microscope. Two typical heteroduplexes are shown in Fig. 2a. Three single-stranded regions of nonhomology were observed, two deletion-insertion loops (DI-1 and DI-2) and one substitution bubble (S). Preliminary calculations showed that the two single-stranded DI loops were most reasonably assigned to γ (see below), and therefore the total duplex length of the hybrid molecule plus the length of S was attributed to β . With the duplex length of β , the molecular mass of its DNA and a conversion factor of 2.08×10^6 per μm (ref. 15), the absolute sizes of various segments of DNA in the heteroduplex were readily calculated. The end of the heteroduplex nearest to the DI-1 loop was arbitrarily chosen as the starting point, and the distances derived are shown in Fig. 2b. Loops DI-1 and DI-2 were assigned to γ -*tsr1* because the sum of these loops, 2.0×10^6 , corresponds closely to the 2.3×10^6 difference in the molecular mass of the two bacteriophage DNAs.

The most significant finding in these heteroduplex studies is that over 99% of β DNA is homologous to γ DNA. Only the 0.11- μm S segment fails to hybridise. Conversely, if the sizes of DI-1, DI-2 and the estimated length of β are summed to give the approximate size of γ , it can be calculated that over 91% of γ DNA is homologous to corresponding regions of β DNA. The DI loops were assigned to γ in these calculations. Further evidence of this close relationship is provided by the electrophoretic banding patterns of restriction enzyme digests of the two phage DNAs. DNA from β -*tsr3* and γ -*tsr1* was cleaved with restriction enzymes and the resulting fragments were compared by electrophoresis on Agarose gels¹⁶. The gels show that β and γ have remarkably similar patterns, although in all digests γ DNA displayed one or more bands not found in β DNA (Fig. 3).

The heteroduplex of β and γ DNA establish unequivocally that there is a close evolutionary relationship between these two phages, a conclusion previously suggested by circumstantial genetic, serological and morphological data. The restriction enzyme analysis also strongly



b

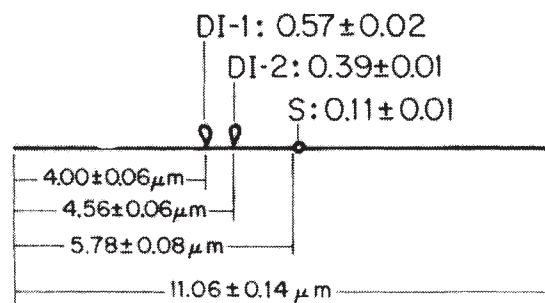


Fig. 2 a, Heteroduplexes between β -*tsr3* and γ -*tsr1*. Heteroduplexes were prepared by mixing 0.1 to 0.2 μg of each DNA in 0.25 ml of a 0.1 M NaOH, 0.01 M EDTA aqueous solution at pH 12.4–12.6 for 10 min, at which time the pH was reduced to 8.4–8.6 by adding 2.0 M Tris (pH 7.1). Formamide, 0.25 ml, was added and the mixture allowed to re-anneal at room temperature for 4–6 h at which time the DNA was spread and rotary shadowed as described¹⁴. All electron microscopy was done with a JEOL Model 100B electron microscope. b, Graphical representation of the heteroduplex.

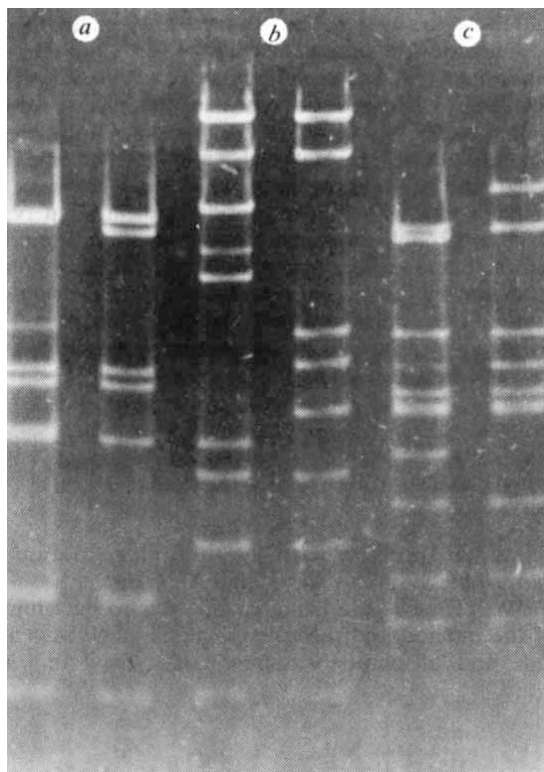


Fig. 3 Restriction enzyme digests after electrophoresis on 0.5% Agarose gels. *a*, *SalI*; *b*, *BAM H-1*; *c*, *Eco RI*. In each case the γ -*tsr1* DNA is in the left lane and the β -*tsr3* DNA in the right lane. One minor band that appears at the bottom of the negative for the *SalI* digest of both γ and β does not appear in this photograph.

supports this conclusion. The high degree of similarity seen in the DNA pattern of the digest is more significant than the differences, since small changes in nucleotide sequences (including single base changes) can alter DNA banding patterns. The presence of one or more minor bands in the Agarose gels (Fig. 3) indicates that there is a small amount of heterogeneity in the digests of γ DNA. Since this heterogeneity was not eliminated by clonal reisolation it is assumed to be intrinsic to the γ population. The reason for this heterogeneity is not clear, but the limited nature of this variation does not affect the primary conclusions of this paper.

Data from the restriction enzyme digests also confirm the molecular mass of β and γ DNA obtained by direct measurement. Standards were used to calculate the approximate masses of each fragment in the digest and these were summed to provide an independent estimate of the size of the two phage DNAs. In the three digests used in these calculations, β -*tsr3* varied in size from 21 to 22×10^6 and γ -*tsr1* varied from 22 to 23×10^6 . Although these figures are slightly smaller than those reported above from direct measurements, they are confirmatory, particularly since the summation of fragment weights is less precise than calculations from contour length. Significantly, in each enzyme digest γ DNA was consistently 1 to 2×10^6 molecular masses larger than β . This closely approximates the size differences calculated from the direct measurements.

On the basis of previous observations (see above), a large degree of homology was expected between β -*tsr3* and γ -*tsr1*. However, it is of particular interest that though these phages are heteroimmune their DNAs are 99% homologous. This suggests that parental β and γ phages are either recent evolutionary derivatives, one from the other, or recently shared a common ancestor. If so, then heteroimmunity must have arisen either through a small number of muta-

tions or through recombination with a distantly related corynephage. Strong selective pressure for genes outside the immunity region would also account for the high degree of homology, but the extent of homology makes it less likely that this is the primary conserving mechanism.

It is not possible to identify the regions of nonhomology between β and γ phages without appropriate mutants. Because these phages are heteroimmune the immunity region is an obvious candidate for the region of nonhomology seen in the substitution bubble. Although β phage is *tox*⁺ and γ is *tox*, it is known that γ carries at least a portion of the *tox* gene⁸. It is possible that the cryptic nature of the *tox* gene in γ is the result of an insertion of nonhomologous DNA into the *tox* gene. If this were the case it would account for one or both of the loops.

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GREGORY BUCK
NEAL GROMAN
STANLEY FALKOW

Department of Microbiology and Immunology,
School of Medicine, SC-42,
University of Washington,
Seattle, Washington 98195

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1. Freeman, V. J. *J. Bact.* **61**, 675-688 (1951).
2. Groman, N. B. *J. Bact.* **69**, 9-15 (1955).
3. Groman, N. B. & Eaton, M. *J. Bact.* **70**, 637-640 (1955).
4. Groman, N. B., Eaton, M. & Booher, Z. K. *J. Bact.* **75**, 320-325 (1958).
5. Holmes, R. K. & Barksdale, L. J. *J. Virol.* **5**, 783-794 (1970).
6. Holmes, R. K. & Barksdale, L. J. *J. Virol.* **3**, 586-598 (1969).
7. Laird, W. & Groman, N. *J. Virol.* **19**, 208-219 (1976).
8. Laird, W. & Groman, N. *J. Virol.* **19**, 220-227 (1976).
9. Groman, N. & Laird, W. *J. Virol.* **23**, 587-591 (1977).
10. Uchida, T., Pappenheimer, Jr. A. M. J. & Greany, R. *J. biol. Chem.* **248**, 3838-3844 (1973).
11. Davis, R. W., Simon, M. & Davidson, N. *Methods Enzym.* **21**, 413-427 (1971).
12. So, M., Gill, R. & Falkow, S. *Molec. gen. Genet.* **142**, 239-249 (1976).
13. Gill, D. M., Uchida, T. & Singer, R. A. *Virology* **50**, 664-668 (1972).
14. Lai, C. J. & Northaus, D. *J. molec. Biol.* **89**, 179-193 (1974).
15. Lang, D. *J. molec. Biol.* **54**, 557-565 (1970).
16. McDonnell, M. W., Simon, M. N. & Studier, F. W. *J. molec. Biol.* **110**, 119-146 (1977).

CIDNP in tyrosyl protons of luliberin

We report here on the possibility of extending CIDNP (chemically induced nuclear magnetic polarisation)-assisted NMR studies of the tyrosyl unit to medium sized peptides. Our previous studies indicate that, strong ¹H nuclear polarisation may usually be obtained in phenols in the presence of dyes such as fluorescein or any of its halogenated derivatives, such as rose bengal, erythrosin B or eosin Y, and others excited to their triplet state¹⁻⁴. The polarisation is due to the H atom transfer from the hydroxyl of the phenol ArOH to the triplet excited dye ³D,



and back,



Steps (a) and (b) were shown to be chemically reversible, the nuclear polarisation resulting from the magnetic interactions in spin-correlated radical pairs $\text{ArO}^\bullet + \text{DH}^\bullet$.

We recently reported that the phenolic amino acid tyrosine and some of its simple derivatives as well as small tyrosyl peptides show a similar phenol CIDNP in certain well defined conditions⁵ (Dr R. Kaptein (Groningen) has performed similar

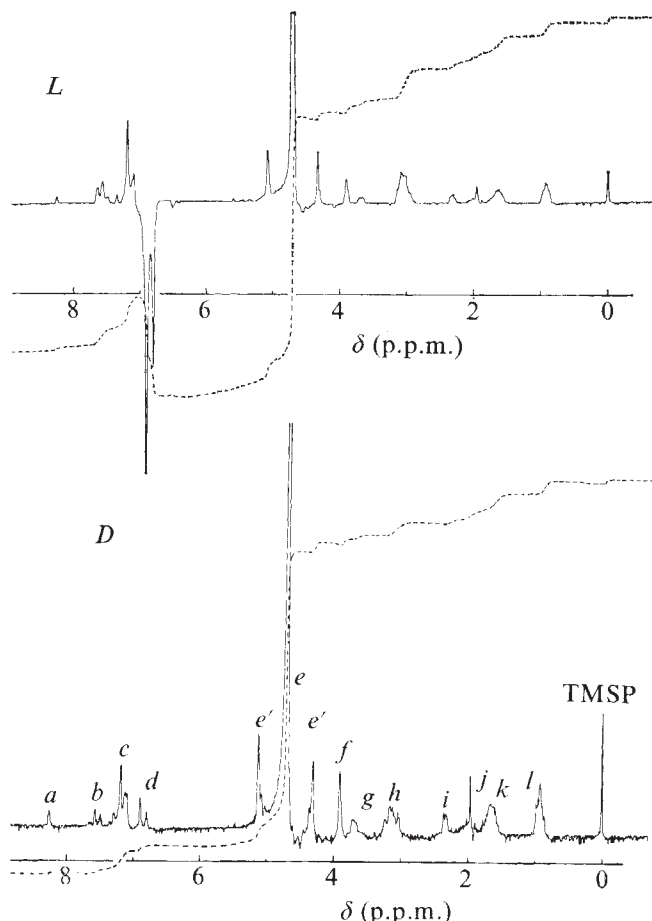


Fig. 1 (—) ^1H 90 MHz FT NMR spectra of luliberin acetate (0.01 M) in D_2O in the presence of fluorescein (2×10^{-3} M). Broken lines (---) give the integration curves. Each spectrum is the result of 256 accumulated pulses. *D*, dark spectrum; *L*, light spectrum (RF pulse preceded by 1 s ultraviolet irradiation period). The protons are assigned according to Vessel *et al.*⁸ *a-d*, aromatic rings; *a*, His; *b*, Trp; *c*, Trp and Tyr meta to OH; *d*, Tyr ortho to OH; *e* and *e'*, HDO impurity; *f*, methylene of Gly- NH_2 ; *g*, Ser methylene; *h*, Tyr, His, Trp and Arg methylene; *i*, Glu and Pro methylenes; *j*, acetate; *k*, Leu and Arg methylenes; *l*, Leu methyl; TMSP, trimethyl silyl propanoic acid, d_4 used as $\delta = 0$ standard.

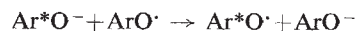
tyrosine CIDNP studies with other unspecified dyes). In our studies, carried out in D_2O solutions in the presence of fluorescein, strong CIDNP effects were obtained on the tyrosine ring protons ortho to the OH group and on the tyrosine methylene protons.

The particular substrate chosen in this investigation of the triplet dye-induced tyrosyl CIDNP in peptides was luliberin^{6,7}, the hypothalamic luteinising hormone releasing factor (LRF). This linear decapeptide of amino acid sequence pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly- NH_2 , was used as the acetate after repeated exchange of its acidic protons with D_2O .

Figure 1 (*L*) shows the effect of ultraviolet irradiation on the proton NMR spectrum of a 0.01 M solution of luliberin in D_2O in the presence of fluorescein (0.002 M), which acts as the H atom photoabstractor. The tyrosyl ring protons ortho to the hydroxyl group (*d*) show a 17-fold negative enhancement *E*. Signal *h*, due to the tyrosyl methylene protons and to methylene protons of His, Trp and Arg⁸ similarly shows a 2-fold enhancement (*A*). The signs of the CIDNP effect for ortho and methylene protons (— and +) are the same as those found in the other phenols¹⁻³, in complete agreement with the analysis

based on the radical-pair model of CIDNP⁹. The *E* and *A* effects in luliberin are similar in magnitude to the effects observed in other tyrosine compounds with the exception of H-Tyr-Tyr-OH, where considerably larger effects were observed (ref. 5 and K. A. M. & C. G., in preparation). The two synthetic derivatives¹⁰ [L-Ala⁶]LRF and [D-Ala⁶]LRF were also studied, and gave almost identical results.

Preliminary results in smaller peptides indicate that tyrosine ^1H CIDNP is strongly pH dependent. This effect should probably be attributed to the nuclear relaxation of the polarised nuclei catalysed by fast degenerate electron exchange processes (* denoting nuclear polarisation)



and



The efficiency of these is due to the short T_1 relaxation times of the paramagnetic species $\text{Ar}^*\text{O}^\bullet$ and $\text{Ar}^*\text{OH}^{*\bullet}$.

Further experiments are being carried out to determine the scope of the method and the role of factors such as structure of peptide and of photoabstractor, and tyrosyl unit accessibility.

K. A. MUSZKAT

Department of Structural Chemistry,
The Weizmann Institute of Science,
Rehovot, Israel

C. GILON

Department of Organic Chemistry,
The Hebrew University,
Jerusalem, Israel

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- Muszkat, K. A. & Weinstein, M. J. *C. S. Chem. Commun.* 143 (1975).
- Muszkat, K. A. & Weinstein, M. *Forster Memorial Volume, Z. phys. Chem.* 101, 105 (1976).
- Muszkat, K. A. & Weinstein, M. J. *C. S. Perkin II*, 1072 (1976).
- Muszkat, K. A. *Chem. phys. Lett.* 49, 583 (1977).
- Muszkat, K. A. *J. C. S. Chem. Commun.* 872 (1977); Muszkat K. A. & Gilon, C. *Biochem. biophys. Res. Commun.* (in the press).
- Schally, A. V. *et al. Biochem. biophys. Res. Commun.* 43, 393 (1971).
- Amoss, M. *et al. Biochem. biophys. Res. Commun.* 44, 205 (1971).
- Wessel, P. L. *et al. J. Chem. Soc. Perkin II*, 1691 (1973).
- Muys, L. T. (ed.) *Chemically Induced Magnetic Polarization, Nato Advanced Study Institutes Series 34*, (Reidel, Dordrecht, 1977).
- Monahan, M. W., Amoss, M. S., Anderson, H. A. & Vale, W. *Biochemistry* 12, 4616 (1973).

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matters arising

Upwelling by icebergs

NESHYBA¹ argues for the importance of iceberg melting below the thermocline in introducing nutrient rich water into the surface layer. I believe she overrates this effect. She assumes that icebergs melt as a single piece and does not even consider calving as part of the deterioration process. Icebergs that are protected from wave action by sea ice are observed to deteriorate little. Icebergs begin to undergo rapid deterioration when they emerge from the sea ice into the open sea². Wave action progressively melts a groove at the waterline by turbulent mixing. I have observed these grooves to have a depth of up to 7–10 m. The sides of the iceberg then fail, producing a large number of small icebergs and 'growlers' and much fine brash. These small icebergs and growlers, which in most cases would be less than 30 m long and have a maximum draft of 20–25 m, are subject to the same erosional forces. Although no hard figures exist at this time, I would estimate that more than 80% of the melting of an iceberg takes place in a surface layer no deeper than 20 m which is in most cases shallower than the main thermocline.

R. Q. ROBE

US Coast Guard Research and Development Center,
Avery Point, Groton,
Connecticut 06340

1. Neshyba, S. *Nature* 267, 507–508 (1977).
2. Robe, R. Q., Kollmeyer, R. C. & Maier, D. C. *Nature* 267, 505–506 (1977).

Cadmium in seabirds

I FEEL that the article on cadmium in marine animals by Bull *et al.*¹ contains a number of misstatements. Seabirds do not generally contain higher residues than marine invertebrates; as long ago as 1956 Mullin and Riley² reported that molluscs may contain up to 500 mg of cadmium per kg in their digestive organs and renal glands, a level not yet equalled by birds, and Holdgate³ reported much the same levels in healthy guillemots *Uria aalge* from British waters as Bull *et al.*¹ do for other marine animals.

Anderlini *et al.*⁴ only investigated six, not seven, bird species, and then only looked at the eggs, which contain little cadmium, for three of them; they included birds from the Atlantic as well as the Pacific and Antarctic. In the three

species where they tested the livers, the most novel finding was perhaps not so much the presence of a mean level of 53 mg of cadmium per kg in ashy petrels *Oceanodroma homochroa* from the polluted coast of California, as that of nearly half as much (means of 20–28 mg per kg) in the livers of snow petrels *Pagodroma nivea* resident in the Antarctic pack ice and Wilson's petrels *Oceanites oceanicus* breeding in Antarctica and migrating into different northern oceans. Similarly, the main point of interest about the puffins *Fratercula arctica* examined by Parslow *et al.*⁵, only one of which was taken alive at a colony as reported by Bull *et al.*¹, was that the two healthy birds from remote west coast colonies contained much more cadmium than the six mainly found washed up on beaches from the east coast. They also reported surprising levels in a herring gull *Larus argentatus* (12 mg per kg) and a Manx shearwater *Puffinus puffinus* (24 mg per kg) as well as the fulmar *Fulmarus glacialis* liver level of 159 mg per kg actually quoted by Bull *et al.*¹ which is higher than any reported by the latter from the same laboratory when they claimed 'considerably higher levels than any previously found'; the ashy petrel mean is in fact also near their highest liver level.

In addition to their references to past evidence for the normal occurrence of high cadmium levels in pelagic seabirds, Bull *et al.*¹ also seem a little careless in their discussion of their own observations on the occurrence of cadmium in sea-skaters *Halobates micans*. They state 'some of the areas where the higher cadmium residues occur, for example off the coast of West Africa,

have little industrial activity, but have upwelling currents of cold water which bring to the surface nutrients, and possibly cadmium too. Areas of ocean upwelling are known to be important wintering locations for pelagic seabirds.' Leaving aside the question whether the bird species quoted do in fact winter in upwelling areas, the map published by Bull *et al.*¹ shows no particular concentration of cadmium in *Halobates* in such areas, but in fact an apparently random distribution of levels above 25 mg per kg throughout the area sampled including the centre of the Sargasso Sea, which is more celebrated for sinking water than upwelling.

Bull *et al.*¹ attribute to me the statement that individual birds are becoming increasingly contaminated with cadmium through feeding in areas of local pollution around the British coast. In point of fact, I ended the paragraph which I devoted to cadmium⁶ 'it would appear that like some other marine animals pelagic seabirds may accumulate exceptional amounts of this element, whose effects are still obscure'. This statement attributed to me comes from the next paragraph summarising observations of heavy metals in general, though as Holdgate³ reported up to 13 mg per kg cadmium in dead guillemots washed up in western Britain in the autumn of 1969 when the level in normal birds was low, it seems possible that there may be some local pollution with cadmium as well as other metals of birds frequenting our offshore waters. If this is the case with either cadmium or any other pollutant it seems doubtful whether it will be detected by looking at birds from remote breeding stations which feed elsewhere, rather than at individuals dying on the spot. It would also be helpful if Monks Wood Experimental Station could place all their other estimations of pollutants on record.

W. R. P. BOURNE

3 Contlaw Place,
Milltimber, Aberdeen, UK

Matters Arising

Matters Arising is meant as a vehicle for comment and discussion about papers that appear in *Nature*. The originator of a Matters Arising contribution should initially send his manuscript to the author of the original paper and both parties should, wherever possible, agree on what is to be submitted. Neither contribution nor reply (if one is necessary) should be longer than 300 words and the briefest of replies, to the effect that a point is taken, should be considered.

1. Bull, K. R., Murton, R. K., Osborn, D., Ward, P. & Cheng, L. *Nature* 269, 507–509 (1977).
2. Mullin, J. B. & Riley, J. P. *J. mar. Res.* 15, 103–122 (1956).
3. Holdgate, M. W. *The Seabird Wreck of 1969 in the Irish Sea* (Natural Environment Research Council, London, 1969).
4. Anderlini, V. C., Connors, P. G., Risebrough, R. W. & Martin, J. H. in *Proc. Symp. Conserv. Prob. Antarctica* (ed. Parker, B. C.) 49–62 (Allen Landrace, Kansas, 1972).
5. Parslow, J. L. F., Jefferies, D. J. & French, M. C. *Bird Study* 19, 18–33 (1972).
6. Bourne, W. R. P. in *Marine Pollution* (ed. Johnston, R.) 403–502 (Academic, London, 1976).

Transmitter release statistics are meaningful

BARTON and Cohen¹ recently attempted to show that statistical analyses of neurotransmitter release, especially those based on a simple binomial model, are of dubious validity. Given their point of view, the authors might have asked: are any statistics meaningful? Every statistical analysis is based on certain assumptions, some purely formalistic, some containing empirical elements. If the statistician assumes an underlying binomial distribution then he tacitly assumes that there are a number of units, each of which may act independently in two ways with a certain probability for each way, the respective values for these probabilities being the same for all units. A binomial assumption was first satisfactorily applied to transmitter release at the crayfish neuromuscular junction². In general terms such an analysis assumes units involving a certain class, *C*, of probability distributions. The basic parameters of this class are (1) the form of dependence of the units; (2) the number of units, *n*; (3) the probabilities of release, $p_1, p_2, \dots, p_n$. The binomial is the simplest subclass of the whole with independence, *n* constant and $p_1 = p_2 = \dots = p_n$. Each element of this subclass can be approximated in many ways by more complicated elements of *C*, but is there any value in that when the simplest analysis suffices?

For example, in the case of falling bodies a random exponent, distributed about 2.00001, would serve as well as the classic quadratic relation; orbits other than a simple ellipse could suffice to describe the path of the earth around the sun.

These examples illustrate the 'principle of parsimony' which postulates that one's observations be described by as simple a model as possible. Experimental observations which cannot be described by the simplest model can then be used for gaining insight into the underlying processes in the real world and are described by, stepwise, more complex models (for example, the branch-block model proposed to explain experimental findings at the frog neuromuscular junction³).

The computed example of Barton and Cohen presents the result of a concatenation of binomial trials with a wide range of *p* values. However, it is difficult to conceive that over 100 quanta with such low release probabilities ($p < 0.01$ in over 70% of quanta) would result in so few failures of release (their Fig. 1b shows only 2–3 failures). Nonetheless, in the result as presented, the simulated and predicted curves would seem to be different; indeed, the χ^2 value quoted indicates that, had they simulated about 250 trials instead of 100, a 5% level of significance would have been achieved with the same relative differences between

the two histograms. This emphasises the importance of an adequate number of samples for satisfactory analysis of transmitter release.

We find the paper of Barton and Cohen a negative one, which makes no contribution to the real need, of which our own experimental results have made us particularly aware. This need is for a comprehensive theoretical treatment of particular generalisations of the binomial model, of which the branch-block model is simply one. Only then will interpretation of experimental results be soundly based; but without the experiments science can become merely a meaningless play with models.

E. HANSERT
A. WERNIG
J. J. CARMODY

Max-Planck-Institut für Psychiatrie,
D-8000 München 40,
Kraepelinstraße 2, FRG

1. Barton, S. B. & Cohen, I. S. *Nature* **268**, 267–268 (1977).
2. Johnson, E. W. & Wernig, A. J. *Physiol., Lond.* **218**, 757–767 (1971).
3. Carmody, J. J. & Wernig, A. *Pflügers Arch. ges. Physiol.* **R32** (1977).

BARTON AND COHEN REPLY—We indicated¹ that many different *p* distributions can lead to histograms of the quantal content which are indistinguishable from the binomial predictions. There are a number of reasons for rejecting the plea of Hansert, Wernig and Carmody that only the 'simplest analysis' should be considered. As we mentioned in our letter, the binomial assumption conflicts with at least two experimental observations. First, quantal contents greater than the binomial estimate of *n* have been recorded at the crayfish neuromuscular junction; this is equivalent to a release of more than the total number of available quanta. Second, the timing of spontaneously released quanta is clustered, implying temporal or spatial non-uniformity of the release process. It is not obvious how the branch-block modification can explain this property of spontaneous release.

The implications of statistical analyses of quantal release are critically dependent

on the assumed probability distribution. *n* is generally assumed to represent an extensive property of the release process and *p* an intensive one. However, if the binomial assumption is false then a change in the *p* distribution of a fixed number of quanta would be interpreted as a change in both *n* and *p*, or even as a change in *n* alone.

The principle of parsimony is used to select the simplest of a set of equivalent hypotheses². However, this principle is irrelevant because the predictions of the assumptions in question are different and experimental distinction of these hypotheses is possible.

We would like to clarify three other points raised by Hansert *et al.* First, there are only two failures of release when 70% of the quanta have $p < 0.01$ because those quanta with high *p* values effectively prevent large numbers of failures. If a single quantum had $p = 1$ then no failures would ever occur. Second, we described a simulation of 100 trials—a sample size often used by some workers³—but simulations of 1,000 trials produced similar results (see Table 1). It is incorrect to assume, as Hansert *et al.* did, that the relative shape of the quantal-content histogram remains the same when the sample size is increased. After publication of our letter it was brought to our attention that Brown, Perkel and Feldman⁴ had performed a similar analysis in 1976 and had reached the same conclusions that we did; they used 1,000 trials and studied the effects of temporal and spatial variations in *n* or *p*. The distributions of quantal contents remained indistinguishable from the binomial predictions even at this high sample size. Third, we agree that a general theoretical treatment would be valuable, but the successful development of this seems unlikely without more data.

These arguments suggest to us that the assumption of binomial release is unlikely to be valid, despite its mathematical simplicity. We believe that the contribution of our letter can be determined only by its success or failure in stimulating new experiments designed to differentiate between the various hypotheses.

STUART B. BARTON

University Laboratory of Physiology,
Oxford, UK

IRA S. COHEN

Department of Physiology
and Biophysics,
Health Sciences Center,
SUNY, Stony Brook, USA

Table 1 The results of six simulations

χ^2	Degrees of freedom	<i>P</i>
5.421	7	0.61
0.924	5	0.97
6.921	6	0.34
3.094	8	0.93
8.172	6	0.23
9.305	6	0.16

The distribution of the release probability of the 100 quanta was generated in the same way as that in Fig. 1a of our earlier letter¹ ($\bar{z} = -5$, $\sigma_z^2 = 2$). 1,000 trials were performed in each simulation. The χ^2 value compares the simulated distribution of quantal contents with the best-fitting binomial distribution. The *P* values give the probability that a value of χ^2 greater than the observed value can arise by chance.

1. Barton, S. B. & Cohen, I. S. *Nature* **268**, 267–268 (1977).
2. Popper, K. R. *The Logic of Scientific Discovery* (Hutchinson, London, 1959).
3. Bennett, M. R. & Fisher, C. J. *Physiol., Lond.* **271**, 673–698 (1977).
4. Brown, T. H., Perkel, D. H. & Feldman, M. W. *Proc. natn. Acad. Sci. U.S.A.* **73**, 2913–2917 (1976).

reviews

Phylogeny of immunity

Nicholas Cohen

Immunity in Evolution. By John J. Marchalonis. Pp.xx+316. (Edward Arnold: London, 1977.) £10.

RECENT years have witnessed a surge of quality research and heightened theoretical interest in the plethora of problems posed by the phylogeny of immunity. Within the past two years, this activity has led to the birth of the journal, *Developmental and Comparative Immunology*, and to the formation of the Division of Comparative Immunology within the construct of the American Society of Zoologists. This activity has also been translated into several reviews, publication of the proceedings of four substantive international symposia, and, as well might be expected, the appearance of three monographs. Dr Marchalonis' labour of love, *Immunity in Evolution*, is the most recent single-authored volume to appear. Fortunately, for readers and authors (and publishing companies), redundancy has been minimised, since each monograph vividly mirrors its author's unique interest, expertise, and personality.

Marchalonis devotes a full 10 chapters to a discussion of the evolution of immunoglobulin structure and function, an area in which his first-hand experience and insight is well-known. Following a detailed review of the structure of mammalian immunoglobulins and the arrangement of immunoglobulin genes in mammals, Marchalonis digests, in a well organised and well written fashion (and in separate chapters) the large body of literature dealing with invertebrate humoral mediators of defense; the phylogenetic emergence of IgM; light chains; the distribution of immunoglobulins distinct from the IgM class; variable regions; antigenic studies of immunoglobulins of eutherian mammals; molecules that might possibly be related to immunoglobulins; and the origins of antibody diversity. His text is more than a paraphrasing of the original papers; it is an assimilation that focuses on concepts and enigmatic problems as well as on facts.

Although Marchalonis does not deal with the evolution of the cellular basis of immunity with the same detail and

perceptive depth that he devotes to humoral immunity, he does critically discuss many of the salient aspects of quasi-immune recognition and effector systems of invertebrates; the emergence of lymphoid cells and organs; the phylogenetic distribution of cell-mediated immune functions; memory and tolerance; and manipulations of the immune systems of ectothermic vertebrates with temperature. It is in these chapters, however, that one shortcoming of the book emerges, a blemish that is revealed only because of the very vitality of the field with which it deals. Although fewer than 2% of the more than 700 cited references were published in 1975 (none were later), it is precisely during the past three years that many of the queries commendably raised by Marchalonis that bear, for example, on the evolution of lymphocyte heterogeneity, the evolution of the major histocompatibility complex, cell cooperation, and the selective effects of temperature on a subset of T cells, have been approached and partly answered. It is also during this period that major concepts in cellular immunology have emerged that may have striking ramifications in understanding the evolution of immunity

(for example, associated recognition). In short, the time from submission and revision of the manuscript to its publication this year was too long.

Fortunately, it is relatively easy for the interested reader to remedy this understandable problem. He or she need only peruse one of the recent photo-offset published proceedings of the symposia held in 1975-77 (*Developmental Immunology*, *Am. Zool.*, **15**, 3-213; 1975; *Immunologic Phylogeny*, ed. W. H. Hildemann and A. A. Benedict, *Adv. exp. Med. Biol.*, **64**, Plenum: New York; 1975; *Phylogeny of Thymus and Bone-Marrow-Bursa Cells*, ed. R. K. Wright and E. L. Cooper, North-Holland: Amsterdam; 1976; *Developmental Immunobiology*, ed. B. M. Solomon and J. D. Horton, North-Holland: Amsterdam; in press) to update their information. Marchalonis' book has provided many of the provocative questions and has predicted several experimental scenarios; these symposia provide at least some of the answers. □

Nicholas Cohen is Associate Professor of Immunology at the University of Rochester School of Medicine and Dentistry, Rochester, New York.

Liquid semiconductors

Liquid Semiconductors. By Melvin Cutler. Pp. 226. (Academic: New York and London, 1977.) \$19.50; £13.85.

PHYSICISTS weaned on the nearly-free-electron theory of solids find it satisfying, if at first surprising, that the properties of most liquid metals can be treated within this approximation. As long as the scattering of the conduction electrons by the non-crystalline arrangement of atoms is weak, perturbation theory can be used, as for example in Ziman's theory of electrical conductivity. It seems that many of the recently discovered metallic glasses can also be described in these terms. However, if the scattering is so strong that the mean free path becomes comparable to the average lattice spacing, then perturbation theory is no longer adequate and new approaches must be sought. This is the situation in certain liquid

metals and in all amorphous and liquid semiconductors.

Concepts required for an understanding of the physics of amorphous and liquid semiconductors have evolved over the past ten years. It is clear that many properties are determined by the short range or local configuration of atoms—that is, bond length, bond angle and coordination number. These determine the main features of the distribution in energy of the electronic states. It is quite proper, therefore, that the author of this book should stress the tight-binding or molecular-bond model when interpreting experimental behaviour. Electrical conductivity, Hall effect and other transport properties demand special treatment, since the concept of well defined wave vectors for the electrons becomes inappropriate and the relaxation time approximation breaks down. The author describes the

current level of understanding of these properties, calling for the most part on models developed to describe amorphous semiconductors. For liquids, of course, further difficulties arise. The local atomic configuration and hence the electronic structure changes not only with time but also with temperature. Thus, if measurements are made as a function of temperature, one has to deal with a different material at each temperature.

The field is not short of experimental results and these are presented in several chapters, one devoted to physical properties, one to physico-chemical properties and metallurgical properties, and two others to specific alloys. The book is valuable for this collection alone. It is not surprising to find Ti-Te alloys discussed extensively, since this system has not only been exhaustively investigated by the author himself, but also exhibits so many of the diverse and interesting properties characteristic of liquid semiconductors. Experimentalists will appreciate the short chapter on techniques.

It behoves a reviewer to draw attention to faults lest it be thought that he is a friend of the author (which he is) or else in league with the publishers (which he is not). My only criticism (apart from an unfortunate interchange of the words band and bond between two section headings in the list of contents), is that tantalising extracts of theory and models, described in detail in later chapters, are interspersed with experimental data presented earlier in the book; yet if the reader tries the theoretical chapters first, he is confronted with tantalising extracts of data. One has to learn, however, to find one's way around any book of this kind and specialists in the field will certainly have no trouble.

The price came as a pleasant surprise (by today's standards) and the book *has* to be good value for such an admirable condensation of a difficult subject by a leader in the field.

E. A. Davis

E. A. Davis is Lecturer in Physics at the University of Cambridge, UK.

Group theory or general relativity

Group Theory and General Relativity. By Moshe Carmeli. Pp. 391. (McGraw-Hill: New York and Toronto, 1977.) £16.85.

THERE is no doubt that a textbook which links together the topics of group theory and general relativity is very welcome. The jacket of this book proclaims that it is the first book on "the applications of group theory to general relativity", but I have some reservations about whether this is an appropriate description. Reading the book gives the impression of two research monographs grafted together: one on group theory, the other general relativity. This does not mean that the contents are not useful, but some stronger motivation for the conjunction of topics would have been desirable.

Field theoreticians are well used to relying heavily on group theoretical techniques, particularly with the recent activity in the area of gauge theories. When it comes to general relativity, group techniques are less powerful because of the absence in general of metric-preserving global diffeomorphisms. Of course, interest centres on special model spacetimes with particular symmetries, and group theory has long played an elegant and useful role in elucidating and classifying their properties. More general classes of

spacetimes, however, can be fruitfully investigated using group methods. Especially important are those that admit asymptotic symmetry groups, and the formulation of concepts such as asymptotic energy momentum, and multipole moments of the gravitational field for isolated bodies relies heavily on the analysis of these groups. In addition to this, much attention has recently been directed to gauge theories in curved spacetime, in which the gauge group continues to play an important part.

This book covers much of the material relevant to these applications, but in a somewhat shapeless form. Readers who have no previous knowledge of one or the other (or both) central topics may find the presentation a little stark with essential facts simply run off. I would recommend some previous background reading, at least for the group theory, from the books of Naimark or Gel'fand *et al.* These are recommended by the author in his introduction, and Carmeli's treatment is closely similar to these.

The first six chapters contain little general relativity, dealing extensively with the representations of the rotation and Lorentz groups. Chapter 3 treats the spinor representations of the Lorentz group. Chapters 4, 5 and 6 tackle in great depth the principal, complementary and complete series of representations of $SL(2, \mathbb{C})$.

The author's discussion of relativity begins with chapter 7. The treatment is standard and traditional, and no attempt seems to have been made to

present the material in any especially novel or enlightening way, or to exploit many of the techniques of modern differential geometry. It is here that one most feels the need for stronger links with the group theory and motivating remarks. The author goes on to develop a detailed discussion of the spinor formulation of general relativity, and it is particularly useful that this approach, advocated by Roger Penrose, and for so long in widespread use among relativists, is dealt with in an integrated fashion in a student textbook. It is frustrating, though, that many useful results of spinor calculus are relegated to the exercises.

In chapter 9, Carmeli takes up the subject of gauge theories, and after a preliminary discussion of electromagnetic and Yang-Mills fields attempts to formulate general relativity also as a gauge theory. The Newman-Penrose formalism is obtained from a gauge theory approach, though perhaps its physical relevance could have been better described. The Goldberg-Sachs theorem is proved in chapter 10, but its importance is not made transparent.

The final two chapters of the book deal with solutions of the field equations of general relativity and the Bondi-Metzner-Sachs group. There are also several appendices. Once again, all the details are competently collected together, but the uninitiated reader may have difficulty in understanding their wider importance or relevance. Perhaps these people would do well to consult the final paragraph of the Introduction which contains a quotation of Abdus Salam, who learned group theory from Racah: "After attending these lectures I thought this is really too hard; I cannot learn this; one is hardly ever likely to need all this complicated matter. I was completely wrong".

In spite of the specific criticisms concerning the presentation, I find this book a timely addition to the many research papers on these two most important topics. It should prove useful as a teaching text and, at least in conjunction with some background material, it could be used as a course book for either group theory for physicists, or more specifically for relativity courses. The style is straightforward and information retrieval easy. Many exercises are given with each chapter and an extensive bibliography is added. It is elegantly produced, and at £16.85 is not over-expensive for a mathematical book of this length.

Paul Davies

Paul Davies is Lecturer in Mathematics at King's College, University of London, UK.

Coastal zone ecology

The Coastline. Edited by R. S. K. Barnes. Pp. 356. (Wiley: Sussex, New York and Sydney, 1977.) £12.50; \$24.50.

By a nice piece of timing, the publication date of this book coincided with the First European Ecological Symposium on Ecological Processes in Coastal Environments at Norwich, where it was exhibited by the publishers. It certainly caught the attention of the conference participants who seemed to like what they saw; so did I. Having now had the opportunity to read it in some detail, my opinion has not changed.

The stated purpose of the book is to contribute to our understanding of the coastline's ecology and physiography in relation to land-use, management and the pressures to which it is subject. To meet this objective, the editor has brought together a team of contributors who have done an admirable job in condensing a relatively large amount of information into one very readable volume. After an introductory chapter on problems and pressures, there follow thirteen chapters, each concerned with one fairly uniform group of habitats. Seven chapters describe predominantly aquatic environments (lagoons, estuaries and foreshores) and six cover terrestrial environments (reclaimed land, cliffs, dunes and shingle). A common structure has been adopted for each chapter: a summary of the ecological and geomorphological processes, characteristic features and pressures, recommendations for methods of study, particular uses, and finally suggestions for conservation and management policies. Coastline management is dealt with in more detail in a concluding chapter.

Many of the examples given are of British coastal areas, but others are from mainland Europe and some from as far afield as Australia. The diagrams which illustrate the various sections are generally good but the reproduction of the (fortunately few) photographs is very poor. There are errors, but those I found are mostly minor spelling mistakes. However, inaccuracies do occur: for example, on p144, it is erroneously stated that the Nature Conservancy Council do not own land. I consider that there are also some errors of omission. Current proposals for energy generation from a Severn Barrage or wave energy converters are not included; the effect of either on the coast would probably be considerable.

Although thermal pollution is mentioned, the possible damaging effects on plankton by its entrainment in cooling water systems is not discussed. The introduction of alien species, which has already led to a great many changes in some ecosystems, is dealt with rather superficially. It would have been valuable if some developing ideas about various ecosystems were included, such as the probable part played by diatoms and some invertebrates in stabilising sediments by their mucus secretions. It is surprising how little is said about ecological and conservation evaluation of coastal ecosystems, surely an essential step in formulating any management proposals. Perhaps more would have been included on this subject had *A Nature Conservation Review* (for review, see *Nature*, 269, 271, 1977) been published a few years earlier.

As stated in the concluding chapter, we still have to reply heavily on a descriptive approach to many ecosystems, as not enough is known to quantify them; we still have some way to go before we can be confident in our predictive capabilities as coastal managers. In relation to this, it would have been valuable to have had an expansion of the last chapter which concerned itself more fully with an appraisal of the state of the art. Nevertheless, I strongly recommend this book to those concerned with coastal management as well as to anyone seeking a lead into the study of this fascinating zone.

R. Mitchell

R. Mitchell is a Marine Biologist with the Chief Scientist's Team at the Nature Conservancy Council, Huntingdon, UK.

Atmospheric highlights

Earth's Aura. By Louise B. Young. Pp. 305. (Alfred A. Knopf: New York, 1977.) \$12.95.

THIS is a book about some of the highlights of the history of atmospheric exploration, about the effect of man's activities on the atmosphere and about the effect of climate on man. In the first chapter we are taken up with pioneering balloonists rising to unbelievable heights in flimsy baskets as the main structures of the various regions of the atmosphere are introduced. The second and third chapters introduce us to clouds and cloud-seeding experiments, and describe thunderstorms by recounting the drama of Franklin's classic kite experiment and the experience of a German glider pilot caught in a cumulonimbus. The delicate balances which control some of the atmosphere's chemistry (including the ozone problem) are introduced in the next three chapters. The enormous variety of winds, jet streams and waves come next; then optical phenomena, the solar wind and the atmospheres of the planets are dealt with in the same dramatic style. Modern speculations about the way the climate may change are discussed in a final chapter.

It is a fascinating account written in an enthusiastic, dramatic, sometimes gripping, style. The material has been effectively selected so that the book is not only very readable but also informative. Some beautiful colour pictures from space are included in the collection of ten illustrations in the centre of the volume.

Both the general reader and the

specialist will be left with a new perspective on the environment and a new enthusiasm for the continuing quest. "Weather is probably the most dominant factor in determining the quality of human existence", we are reminded, "but although we have set foot on the Moon, have identified organic molecules in the tenuous dust of interstellar space and measured the speed of galaxies a billion light years away, we still do not understand the intricate interplay of forces that move the earth's weather".

On the whole, the book presents the present state of knowledge in an accurate way. The emphasis of one or two of the chapters, however, tends to leave the reader with a misleading impression. One might have wished for instance that alongside the dramatic description of the 1952 London fog, the far-reaching changes brought about by the 1957 Clean Air Act might have been described. Too much attention is also given to the rather speculative area of the influence on the climate of sunspot cycles and the like, whereas virtually no mention is made of the ocean/atmosphere interaction and feedback processes involving the Arctic or Antarctic ice which are probably of much greater importance.

The cover describes the book as science writing at its best: the adventures and discoveries of the past, the problems that still face us. I certainly enjoyed reading it; it must be one of the best popular science books which deals with the atmosphere.

Further, for a well printed book of 700 pages and some colour illustrations, the book is moderately priced.

J. T. Houghton

J. T. Houghton is Professor of Atmosphere Physics at the Clarendon Laboratory, Oxford, UK.

Recent scientific and technical books

Biology

- BARKER, A. N., WOLF, J., ELLAR, D. J., DRING, G. J., and GOULD, G. W. (edited by). *Spore Research 1976*. Vol. 1: Pp.lxvi+1-420. ISBN-0-12-078701-6. £14; \$27.35. Vol. 2: Pp.lxvi+421-915. ISBN-0-12-078702-4. £16; \$31.25. (London and New York: Academic Press, a Subsidiary of Harcourt Brace Jovanovich, Publishers, 1977.)
- BEIDERBECK, Rolf. *Pflanzenzotoren: Ein Problem der Pflanzlichen Entwicklung*. (Phytologie: Klassische und Moderne Botanik in Einzeldarstellungen.) Pp.216. ISBN-3-8001-3420-9. (Stuttgart: Verlag Eugen Ulmer, 1977.) DM 48.
- BERKALOFF, André, BOURGUET, Jacques, FAVARD, Pierre, et LACROIX, Jean-Claude. *Biologie et Physiologie Cellulaires. I. Membrane Plasmique, etc.* Nouvelle édition entièrement refondue et augmentée. (Collection Méthodes.) Pp.271. ISBN-2-7056-5876-9. (Paris: Hermann, 1977.)
- BROOKHART, John M., MOUNTCASTLE, Vernon B., KANDEL, Eric R., and GEIGER, Stephen R. (edited by). *Handbook of Physiology: a Critical, Comprehensive presentation of Physiological Knowledge and Concepts. Section 1: The Nervous System. Vol. 1: Cellular Biology of Neurons. Part 1: Pp.xxviii+1-718. Part 2: Pp.xxviii+719-1182. ISBN-0-683-04505-9. (Bethesda, Md.: American Physiological Society, 1977. Distributed by The Williams and Wilkins Company, Baltimore, Md.) \$135.*
- BUKHARI, A. I., SHAPIRO, J. A., and ADHYA, S. L. *DNA Insertion Elements, Plasmids, and Episomes*. Pp.xiii+782. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory, 1977.) \$36.
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- FERSHT, Alan. *Anzyme Structure and Mechanism*. Pp.xviii+371. ISBN-0-7167-0188-X. (Reading and San Francisco: W. H. Freeman and Company, 1977.) Hardcover £160; Softcover £4.95.
- GARRETT, J. R., HARRISON, J. D., and STOWARD, P. J. (edited by). *Histochemistry of Secretory Processes*. Pp.x+340. ISBN-0-412-14870-6. (London: Chapman and Hall, 1977. Distributed in the USA by Halsted Press, a Division of John Wiley and Sons, Inc., New York.) £17.50.
- HAZLETT, Brian A. (edited by). *Quantitative Methods in the Study of Animal Behaviour*. Pp.x+222. ISBN-0-12-335250-9. (New York and London: Academic Press, a Subsidiary of Harcourt Brace Jovanovich, Publishers, 1977.) \$12.50; £8.85.
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- KINNE, Otto (edited by). *Marine Ecology: a Comprehensive, Integrated Treatise on Life in Oceans and Coastal Waters*. Vol. 3: Cultivation, Part 3. Pp.xix+1295-1521. ISBN-0-471-48007-X. (Chichester and New York: Wiley-Interscience, John Wiley and Sons, 1977.) £12; \$24.
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Anthropology

- GLOB, P. V. *The Bog People: Iron-Age Man Preserved*. Translated from the Danish by Rupert Brice-Mitford. Pp.200. ISBN-0-571-11079-7. (London: Faber and Faber, Ltd., 1977. First published in England in 1969.) £1.95.

General

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- WHEEDEN, Richard L., and ZYGMUND, Antoni. *Measure and Integral: An Introduction to Real Analysis*. (Pure and Applied Mathematics: a Series of Monographs and Textbooks, Vol. 43.) Pp. x + 274. ISBN-0-8274-6499-4. (New York and Basel: Marcel Dekker, Inc., 1977.) Sfr. 54.

Astronomy

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- BOHME, S., ESSER, U., FRICKE, W., GUNTZEL-LINGNER, U., KRAHN, I., HEINRICH D., SCHMADEL, L. D., and ZECH, G. (edited by). *Astronomy and Astrophysics Abstracts*. (A Publication of the Astronomisches Rechen-Institut Heidelberg, Member of the Abstracting Board of the International Council of Scientific Unions, Vol. 19: Literature 1977, Part 1. Pp. x + 732. ISBN-3-540-08555-6. (Berlin and New York: Springer-Verlag, 1977. Published for Astronomisches Rechen-Institut.) DM95; \$43.70.
- EWERS, Ralph O., and EWERS, Lynda M. *A Universe to Explore: Searching for Structure*. Pp. iv + 92. ISBN-0-03-922123-7. (Toronto and Montreal: Holt, Rinehart and Winston of Canada, Ltd., 1976.) £3.50.
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Physics

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- BURNS, Gerald. *Introduction to Group Theory with Applications*. (Materials Science and Technology.) Pp. xv + 429. ISBN-0-12-145750-8. (New York and London: Academic Press, a Subsidiary of Harcourt Brace Jovanovich, Publishers, 1977.) \$18.50; £13.15.
- CLARKSON, B. L. (edited by). *In association with HAMMOND, J. K., HOLMES, P. J., and KISTNER, A. Stochastic Problems in Dynamics*. Pp. 566. ISBN-0-273-01104-9. (London, San Francisco and Melbourne: Pitman, 1977.) £12.
- COOBLIN, B. *The Electronic Structure of Rare-Earth Metals and Alloys: The Magnetic Heavy Rare-Earths*. Pp. xvi + 656. ISBN-0-12-18815-4. (London and New York: Academic Press, a Subsidiary of Harcourt Brace Jovanovich, Publishers, 1977.) £29.50; \$57.75.
- HENDERSON, S. T. *Daylight and Its Spectrum*. Second edition. Pp. x + 349. ISBN-0-85274-343-2. (Bristol: Adam Hilger, Ltd., 1977.) £12.50 net.
- HOLAH, G. D. (edited by). *Ternary Compounds, 1977*. (Invited and contributed papers from the Third International Conference on Ternary Compounds held at Heriot-Watt University, Edinburgh, 14-15 April 1977.) (Conference Series No. 35.) Pp. ix + 326. ISBN-0-85498-126-8. (Bridford and London: The Institute of Physics, 1977. Published in association with the American Institute of Physics, New York.) £20; \$38.
- LATANISION, R. M., and FOURIE, J. T. (edited by). *Surface Effects in Crystal Plasticity*. (NATO Advanced Study Institutes Series E: Applied Science, Vol. 17.) Pp. xix + 944. ISBN-90-286-0066-3. (Leyden: Noordhoff, 1977.) Dfl. 113; \$45.25.
- MISELL, D. L. (edited by). *Developments in Electron Microscopy and Analysis, 1977*. (Proceedings of the Institute of Physics Electron Microscopy and Analysis Group conference held in Glasgow, 12-14 September 1977. EMAG 77.) (Conference Series No. 36.) Pp. xiii + 441. ISBN-0-85498-127-6. (Bristol and London: The Institute of Physics, 1977. Published in association with the American Institute of Physics, New York.) £11; \$21.
- ODABASI, H., and AKYUZ, O. (edited by). *Topics in Mathematical Physics*. Pp. xi + 279. ISBN-0-87081-072-3. (Boulder, Colorado: Colorado Associated University Press, 1977.) np.
- PAVLIDIS, T. *Structural Pattern Recognition*. (Springer Series in Electrophysics, Vol. 1.) Pp. xii + 302. ISBN-3540-08463-0. (Berlin and New York: Springer-Verlag, 1977.) DM 43; \$19.80.
- QUEISSER, H. J. (edited by). *X-Ray Optics: Applications to Solids*. With contributions by A. Authier, et al. (Topics in Applied Physics, Vol. 22.) Pp. xxi + 227. ISBN-3-540-08462-2. (Berlin and New York: Springer-Verlag, 1977.) DM 76; \$35.
- RILEY, F. L. (edited by). *Nitrogen Ceramics*. (NATO Advanced Study Institutes Series E: Applied Science, No. 23.) Pp. xxi + 694. ISBN-90-286-0697-1. (Leyden: Noordhoff, 1977.) Dfl. 120; \$50.
- SALEH, B. *Photoelectron Statistics, With Applications to Spectroscopy and Optical Communication*. (Springer Series in Optical Sciences, Vol. 6.) Pp. xv + 441. ISBN-3-540-08295-6. (Berlin and New York: Springer-Verlag, 1978.) DM 68; \$31.30.
- SEGRE, Emilio. *Nuclear and Particles: An Introduction to Nuclear and Subnuclear Physics*. Second edition, completely revised, reset, enlarged. Pp. xx + 966. ISBN-0-8053-8601-7. Reading, Mass. and London: W. A. Benjamin, Inc., 1977.) \$29.50.
- SHIMOJI, Mitsuo. *Liquid Metals: An Introduction to the Physics and Chemistry of Metals in the Liquid State*. Pp. xi + 391. ISBN-0-12-641550-1. (London and New York: Academic Press, a Subsidiary of Harcourt Brace Jovanovich, Publishers, 1977.) £16.50.

- TER HAAR, D. *Lectures on Selected Topics in Statistical Mechanics*. (International Series in Natural Philosophy, Vol. 92.) Pp. vii + 124. ISBN-0-08-017937-1. (Oxford and New York: Pergamon Press, 1977.) £7.95.
- WOLF, E. (edited by). *Progress in Optics*, Vol. 15. Pp. xv + 364. ISBN-0-7204-1515-2. (Amsterdam, New York and Oxford: North-Holland Publishing Company, 1977.) Dfl. 125; \$50.95.

Chemistry

- BECKER, Ernest I., and TSUTSUI, Minoru (edited by). *Organometallic Reactions and Syntheses*, Vol. 6. Pp. xi + 314. ISBN-0-306-39906-7(v.6). (New York and London: Plenum Press, 1977.) \$47.40.
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- CORBRIDGE, D. E. C. *Phosphorus: An Outline of Its Chemistry, Biochemistry and Technology*. Pp. x + 455. ISBN-0-444-41661-7. (Amsterdam, Oxford and New York: Elsevier Scientific Publishing Company, 1978.) Dfl. 146; \$59.60.
- DAVIES, Mansel (senior reporter). *Dielectric and Related Molecular Processes*, Vol. 3. (Reviews of Recent Developments up to December 1976. A Specialist Periodical Report.) Pp. ix + 259. ISBN-0-85186-525-9. (London: The Chemical Society, 1977. Obtainable from The Chemical Society, Blackhorse Road, Letchworth, Herts.: American Chemical Society, 1155 Sixteenth Street, N.W., Washington, D.C. 20036.) £23; \$46.
- DUNITZ, J. D., HEMMERICH, P., IBERS, J. A., JØRGENSEN, C. K., NEILANDS, J. B., REINEN, D., and WILLIAMS, R. J. P. (edited by). *Structure and Bonding*, Vol. 33: *New Concepts*. Pp. iv + 214. ISBN-3-540-08269-7. (Berlin and New York: Springer-Verlag, 1977.) DM 74; \$32.60.
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- GRAOVAC, A., GUTMAN, Ivan, and TRINAJSTIC, Nenad. *Topological Approach to the Chemistry of Conjugated Molecules*. (Lecture Notes in Chemistry, Vol. 4.) Pp. ix + 123. ISBN-3-540-08431-2. (Berlin and New York: Springer-Verlag, 1977.) DM 18; \$8.30.
- INORGANIC CHEMISTRY: METAL CARBONYL CHEMISTRY. (Topics in Current Chemistry/Fortschritte der Chemischen Forschung, Vol. 71.) Pp. iv + 190. ISBN-3540-08290-5. (Berlin and New York: Springer-Verlag, 1977.) DM 69; \$30.40.
- JOULE, J. A., and SMITH, G. F. *Heterocyclic Chemistry*. Second edition. Pp. viii + 378. ISBN-0-442-30211-8. (London and New York: Van Nostrand Reinhold Company, 1978.) £12 cloth; £5.25 paperback.
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- MARCUS, Y., YANIR, E., and KERTES, A. S. (prepared for publication by). *Equilibrium Constants of Liquid-Liquid Distribution Reactions, Part 3: Compound Forming Extractants, Solvating Solvents and Inert Solvents*. (International Union of Pure and Applied Chemistry, Analytical Chemistry Division, Commission on Equilibrium Data. IUPAC Chemical Data Series, No. 15.) Pp. v + 46. ISBN-0-08-022032-0. (Oxford and New York: Pergamon Press, 1977.) £4.
- MATKOVIČ, V. I. (edited by). *Boron and Refractory Borides*. Pp. viii + 656. ISBN-3-540-08181-X. (Berlin and New York: Springer-Verlag, 1977.) DM 180; \$79.20.
- McAULEY, A. (senior reporter). *Inorganic Reaction Mechanisms*, Vol. 5 (A Review of the Literature Published between January 1975 and June 1976. A Specialist Periodical Report.) Pp. xviii + 454. ISBN-0-85186-295-0. (London: The Chemical Society, 1977. Obtainable from The Chemical Society, Blackhorse Road, Letchworth, Herts.: American Chemical Society, 1155 Sixteenth Street, N.W., Washington, D.C. 20036. U.S.A.) £29; \$58.
- MEDICINAL CHEMISTRY. (Topics in Current Chemistry, Vol. 72.) Pp. iv + 157. ISBN-3-540-08366-9. (Berlin and New York: Springer-Verlag, 1977.) DM 64; \$29.50.
- MILLICH, Frank, and CARRAHER, Jr., Charles E. (edited by). *Interfacial Synthesis*. Vol. 2: Polymer Applications and Technology. Pp. xi + 546. ISBN-0-8247-6373-4. (New York and Basel: Marcel Dekker, Inc., 1977.) Sfr. 190.
- NASS, Leonard I. (edited by). *Encyclopedia of PVC*, Vol. 3. Pp. xiii + 1251-1861. ISBN-0-8247-6471-4. (New York and Basel: Marcel Dekker, Inc., 1977.) \$75.
- OCHIAI, E. *Bioinorganic Chemistry: an Introduction*. (The Allyn and Bacon Chemistry Series.) Pp. xi + 515. ISBN-0-205-05508-7. (Boston, Mass. and London: Allyn and Bacon, Inc., 1977.) £16.95.
- PINES, Herman, and STALICK, Wayne M. *Base-Catalyzed Reactions of Hydrocarbons and Related Compounds*. Pp. xi + 587. ISBN-0-12-557150-X. (New York and London: Academic Press, a Subsidiary of Harcourt Brace Jovanovich, Publishers, 1977.) \$57; £40.45.
- REISEFELD, Renata, and JØRGENSEN, Christian K. *Lasers and Excited States of Rare Earths*. (Inorganic Chemistry Concepts, Vol. 1.) Pp. viii + 226. ISBN-3-540-08324-3. (Berlin and New York: Springer-Verlag, 1977.) DM 64; \$29.50.
- SZANTAY, Cs., and NOVÁK, L. *Synthesis of Prostaglandins*. (Recent Developments in the Chemistry of Natural Carbon Compounds, Vol. 8.) Pp. 267. ISBN-963-05-1303. (Budapest: Akadémiai Kiadó, 1978.) \$16.
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- WEBER, W. P., and GOKEL, G. W. *Phase Transfer Catalysis in Organic Synthesis*. (Reactivity and Structure: Concepts in Organic Chemistry, Vol. 4.) Pp. xv + 280. ISBN-3-540-08377-4. (Berlin and New York: Springer-Verlag, 1977.) DM 64.70; \$29.80.
- WEINSTEIN, Boris (edited by). *Chemistry and Biochemistry of Amino Acids, Peptides, and Proteins: a Survey of Recent Developments*, Vol. 4. Pp. xi + 339. ISBN-0-8247-6604-0. (New York and Basel: Marcel Dekker, Inc., 1977.) Sfr. 128.

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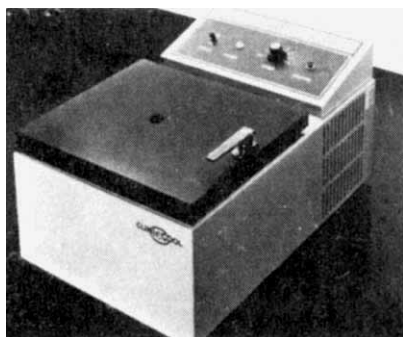
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- HEARN, E. J. *Mechanics of Materials: An Introduction to the Mechanics of Elastic and Plastic Deformation of Solids and Structural Components*, Vol. 1. Pp. xxix + 1-394. ISBN-0-08-018749-8. £10. Vol. 2. Pp. xxii + 395-643. ISBN-0-08-020617-4. £7.50. (Pergamon International Library of Science, Technology, Engineering and Social Studies. International Series in Materials Science and Technology, Vol. 19.) (Oxford and New York: Pergamon Press, 1977.)

what's new – centrifuges

These notes, prepared from material provided by the manufacturers, are intended to give an outline of the range of products on the market. More detailed information may be obtained by circling the appropriate numbers on the Reader Enquiry Card, bound inside the cover. A feature on balances will appear in the 8 May issue.

Damon/IEC. The IEC DPR-6000 high-capacity mid-speed refrigerated centrifuge features a digital speed readout that gives precision to 10 r.p.m., eliminating the need for periodic speed calibration. The DUR-6000 is fitted with a flexible drive shaft, a brush-wear indicator, durable range timer, variable electric brake and precise temperature control. The DPR-6000 attains a maximum speed of 6,000 r.p.m. and maximum force of 7,275g. Maximum capacity is 6 l. The IEC range also includes eight models of bench-top centrifuge and the B-20A and B-60 ultracentrifuges.

Circle No. 51 on Reader Enquiry Card.



The IEC Clini-cool refrigerated bench-top centrifuge

Gallenkamp. The Gallenkamp CFC-301 bench-top centrifuge has a maximum total capacity of 200 ml and has a top speed of 5,000 r.p.m. A built-in timer provides automatic control of running time for up to 20 min, if required. The self-balancing action of the motor ensures that centrifuge tubes need only be balanced by eye. Speed control is provided and choice of swing-out and angle heads is available. Centrifuge CFD-400 is suitable for a large number of routine and research applications, and has a maximum speed of 5,000 r.p.m. and total capacity up to 1,000 ml.

Circle No. 52 on Reader Enquiry Card.

DuPont Instruments (Sorvall). The GLC-3 bench-top model has a capacity of 80 tubes from 10×75- to 12×75-mm at speeds of up to 3,400 r.p.m. and forces of

1,240g. The GLC-2B also takes 80 tubes, providing speeds up to 2,600 r.p.m. and 1,240g. Higher *g* forces are attained by using buckets; the GLC-2B providing speeds up to 6,000 r.p.m. with fixed-angle rotors. The Sorvall RC-5B superspeed refrigerated centrifuge is equipped with a built-in automatic rate controller for soft starts and stops. Temperature, speed and time are programmed automatically. The rate of acceleration can be varied between start and 100 r.p.m. to give optimum acceleration for density gradient runs with vertical and zonal rotors. The Sorvall OTD-50 ultracentrifuge provides speeds up to 50,000 r.p.m. and forces up to 300,000g and the OTD-65 provides 65,000 r.p.m. and 429,297g. Both ultracentrifuges are self-contained: the self-contained cooling unit eliminates the need for external plumbing, filter and valves. The RC-5 superspeed and the OTD ultracentrifuges can be fitted with Sorvall vertical rotors which allow the rapid performance of density gradient separations.

Circle No. 53 on Reader Enquiry Card.

Beckman. The TJ-6 bench-top centrifuge is available with or without refrigeration. The TJ-6 provides speeds of up to 2,700 r.p.m. and 1,520g. Fixed-angle rotors (10×50 ml and 24×15 ml) are rated for 5,700 r.p.m. and 4,470g. The Microfuge B miniature centrifuge holds 48 250- or 400-μl tubes or 18 1.5 ml tapered-bottom tubes. It accelerates almost instantaneously to top speed and can spin down blood cells or protein precipitates in 1 min. Separations for RIA take 3 min. The Model J-21C floor-model centrifuge is designed as a general laboratory centrifuge for initial processing of large particles in large volumes at up to 21,000 r.p.m. and 50,330g. The Beckman range of preparative ultracentrifuges includes the Model L5-40, with a maximum speed of 40,000 r.p.m., 284,000g and the L5-75 which can operate at 75,000 r.p.m., 504,000g. The Beckman Airfuge is a miniature table-top ultracentrifuge capable of rapidly separating small volumes. The rotor holds six 175-μl samples and attains 100,000 r.p.m., 160,000g in 30 s. The Airfuge is powered by standard laboratory air pressure.

Circle No. 54 on Reader Enquiry Card.

MSE Scientific Instruments. The MSE Prepspin 50 preparative ultracentrifuge operates at up to 50,000 r.p.m.

(347,000g) with a maximum capacity of 2,000 ml. The Prepspin features a new overspeed protection system, a wide range of angle and swing-out rotors in aluminium and titanium, full zonal capability, variable acceleration and braking, fast acceleration, automatic oil recirculation, and a brush-wear indicator. Also available are the Prepspin 65 and Prepspin 75 for operation at higher



MSE Prepspin 75

speeds. The Coolspin is a refrigerated centrifuge particularly suited for use in blood transfusion centres. The capacity of this centrifuge is 6 l at over 5,000g; it can accommodate 12 blood bags or carry 288 samples at one time. The Coolspin operates in the temperature range -20° to -40°C : acceleration and braking are variable and acceleration is fast. The range of rotors includes a 4×1,000 ml windshielded swing-out rotor developing 6,140g at 5,200 r.p.m., a two-place head for use in radio-immunoassay and autoanalyser work and the 'A' type zonal rotor. The Hi-Spin 21 is a high-speed 3-l capacity refrigerated centrifuge. The rotor complement includes large-capacity angle rotors such as the 6×500 ml and the 24×15 ml rotors, a continuous action rotor and the HS zonal rotor. To meet the demand for a simple and reliable refrigerated bench-top centrifuge MSE have produced the Chilspin. Chilspin has a maximum speed of 6,100 r.p.m. (6,030g) and uses the same rotors and accessories as the MSE Super Minor.

Circle No. 55 on Reader Enquiry Card.

Hettich. The Hettich EBA 3S is a newly designed bench-top laboratory centrifuge. The spin time can be set to 1–60 min, and the speed of rotation can be pre-selected and monitored on a revolution counter. Angular steel rotors are provided for either four or six buckets. Balancing is not essential because of the elastic suspension of the drive in the housing. The maximum rotor speed is 5,500 r.p.m.

Circle No. 56 on Reader Enquiry Card.